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Vliv malých DNA virů na funkci plasmacytoidních dendritických buněk
Effect of small DNA viruses on function of plasmacytoid dendritic cells

Disertační práce

Školitel: prof. RNDr. Ivan Hirsch, CSc.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Obsah

Seznam zkratek

AIDS	acquired immune deficiency syndrom	syndrom získané imunodeficience
AIM-2	absent in melanoma 2	protein nepřítomný v melanomu 2
AP endonukleáza	apurinic/apyrimidinic endonuclease	apurinní/apyrimidinní endonukleáza
AP-1	activator protein 1	aktivátorový protein 1
APOBEC	apolipoprotein B mRNA editing catalytic polypeptide-like	editační enzym mRNA apolipoporteinu B
ART	active retroviral therapy	aktivní retrovirová terapie
ATF2	activation transcription factor 2	aktivační transkripční faktor 2
BAD-LAMP	brain and DC-associated LAMP-like molecule	LAMP-podobná molekula nalezená v mozku a dendritických buňkách
BCL9	B-cell CLL/Lymphoma 9 Protein	lymfoma protein 9 lymfocytů B
BCR	B cell receptor	receptor lymfocytů B
BDCA-2	blood dendritic cell antigen 2	krevní antigen dendritických buněk 2
BDCA-4	blood dendritic cell antigen 4	krevní antigen dendritických buněk 4
BLNK	B-cell linker	spojovací protein lymfocytů B
BSL3	biosafety level 3	laboratoř s úrovní zabezpečení 3
BST2	bone marrow stromal cell antigen 2	stromální antigen kostní dřeně 2
Btk	Bruton's tyrosine kinase	Brutonova tyrosinkináza
CAMK	calcium/calmodulin-dependent protein kinase	kalcium/kalmodulin dependentní kináza
CARD9	caspase recruitment domain-containing protein 9	protein 9 obsahující doménu rekrutující kaspázy
CARMA1	CARD-membrane-associated guanylate kinase protein 1	CARD-membránově asociovaný protein 1 guanylát kinázy
cccDNA	covalently closed circular DNA	kovalentně uzavřená kruhová DNA
c-FOS	cellular oncogene Fos	buněčný onkogen FOS
c-Jun	proto-oncogene C-Jun	buněčný protoonkogen Jun
CLR	C-type lectin receptor	lektinový receptor typu C
CREB	cAMP responsive element-binding element protein 1	protein 1 vázající se na cAMP responsivní element
DAG	diacylglycerol	diacylglycerol

DAMP	danger-associated molecular patterns	molekuly asociované s poškozením tkáně
DAP12	DNAX adaptor protein 12	adaptorový protein DNAX 12
DCIR	dendritic cell immunoreceptor	imunoreceptor dendritických buněk
EBV	Epstein-Barr virus	virus Epstein-Barrové
EEA1	early endosomal antigen 1	časný endosomální antigen 1
ELISA	enzyme-linked immuno sorbent assay	enzymatická imunologická metoda
ER	endoplasmic reticulum	endoplazmatické retikulum
ERK	extracellular signal–regulated kinase	kináza regulovaná extracelulárním signálem
ESCRT	endosomal sorting complexes required for transport	endosomální sortovací komplex
FcεRIγ	Fc fragment of IgE receptor Ig	Fc fragment IgE receptoru
FOSL1	FOS-like 1 antigen	antigen 1 podobný proteinu c-FOS
HAV	hepatitis A virus	virus hepatitidy A
HBV	hepatitis B virus	virus hepatitidy B
HBeAg	hepatitis B e antigen	e antigen HBV
HBsAg	hepatitis B surface antigen	povrchový antigen S HBV
HCV	hepatitis C virus	virus hepatitidy C
HIV	human imunodeficiency virus	virus lidské imunodeficience
HLA-DR	human leukocyte antigen-DR isotype	lidský leukocytární antigen DR
hNTCP	human natrium/taurocholate cotransport polypeptide	lidský sodíko/taurocholátový kotransportní polypeptid
HSP70	heat shock protein 70	protein teplotního šoku 70
HSV	herpes simplex virus	herpes simplex virus
ICAM-1	intercellular adhesion molecule 1	mezibuněčná adhezivní molekula 1
ICOS	inducible T-cell costimulator	inducibilní kostimulátor lymfocytů T
ICOS-L	ICOS ligand	ligand proteinu ICOS
IFN	interferon	interferon
IFNAR	interferon-α/β receptor	receptor pro interferony typu I
IFNLR	interferon-λ receptor	receptor pro interferony typu III
IgA	imunoglobulin A	imunoglobulin A
IgG	imunoglobulin G	imunoglobulin G
IgM	imunoglobulin M	imunoglobulin M

IKK	inhibitor of nuclear factor kappa-B kinase subunit beta	kináza fosforylující inhibitor NF-κB
IL	interleukin	interleukin
ILT-7	imunoglobulin-like transkript 7	protein podobný transkriptu imunoglobulinů 7
IRAK	IL-1 receptor associated kinase	kináza asociovaná s receptorem pro IL-1
IRF7	interferon regulatory factor 7	interferon regulační faktor 7
ISG	interferon-stimulated genes	interferonem stimulované geny
ISRE	interferon-stimulated response element	interferonem stimulovaný element
ITAM	immunoreceptor tyrosine-based activation motif	aktivační motiv imunoreceptorů založený na tyrosinu
ITIM	immunoreceptor tyrosine-based inhibitory motif	inhibiční motiv imunoreceptorů založený na tyrosinu
IκB	inhibitor NF-κB	inhibitor NF-κB
JAK	janus kinase	janusova kináza
LAIR-1	leukocyte-associated immunoglobulin-like receptor 1	receptor leukocytů podobný imunoglobulinům 1
LAMP1/2	lysosomal-associated membrane protein 1/2	lysozomální membránový glykoprotein 1/2
LFA-1	leukocyte function-associated molecule 1	adhezivní molekula lymfocytů 1
LIR	leukocyte immunoglobulin-like receptor	leukocytární imunoglobulin-podobný receptor
LPS	lipopolysaccharide	lipopolysacharid
LT-β	lymphotoxin beta	lymfotoxin beta
Lyn	kinase Lyn	kináza Lyn
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1	translokační protein 1 asociovaný s lymfomy
MAPK	mitogen-activated protein kinase	mitogenem aktivovaná protein kináza
mDC	myeloid dendritic cells	myeloidní dendritické buňky
MEK	MAPK/ERK kinase 1	MAPK/ERK kináza 1
MHC	major histocompatibility complex	hlavní histokompatibilní komplex
miRNA	microRNA	mikro RNA
MLL-5	mixed-lineage leukemia protein 5	leukemický protein 5
mTOR	mammalian target of rapamycin serine/threonine kinase	savčí cíl rapamycinu serin/threoninová kináza

MyD88	myeloid differentiation factor 88	myeloidní diferenciační faktor 88
NEMO	NF-kappa-B essential modulator	regulační podjednotka inhibitoru NF-κB
NFAT	nuclear factor of activated T-cells	nukleární faktor aktivovaných lymfocytů T
NF-κB	nuclear factor Of kappa light polypeptide gene enhancer in B-cells	nukleární faktor vázající enhancer řetězce Kappa v lymfocytech B
NKp44	natural killer cell P44-related protein	protein buněk NK
NLR	NOD-like receptor	receptor podobný NOD
NUC	nucleos(t)ide analogues	nukleos(t)idové analogy
ODN	oligodeoxyribonucleotides	oligodeoxyribonukleotidy
OPN	optineurin	optineurin
ORN	oligoribonucleotides	oligoribonukleotidy
OX40L	ligand for OX40 receptor	ligand receptoru OX40
PACSIN1	protein kinase C and casein kinase substrate in neurons 1	substrát protein kinázy C a kasein kinázy v neuronech
PAMP	pathogen associated molecular pattern	molekulární vzor asociovaný s patogeny
PBMC	peripheral blood mononuclear cells	periferní krevní mononukleární buňky
PCNA	proliferating cell nuclear antigen	jaderný antigen proliferujících buněk
pDC	plasmacytoid dendritic cells	plasmacytoidní dendritické buňky
pgRNA	pregenomic RNA	pregenomová RNA
PHH	primary human hepatocytes	primární lidské hepatocyty
PI3K	phosphatidylinositol 3-kinase	fosfatidylinositol-3-kináza
PKB	protein kinase B	protein kináza B
PKC	protein kinase C	protein kináza C
PKR	protein kinase R	protein kináza R
PLCγ2	phospholipase Cγ2	fosfolipáza Cγ2
PMA	phorbol myristate acetate	forbol myristyl acetát
PRAT4A	protein associated with TLR4	protein asociovaný s TLR4
PRR	pattern recognition receptor	receptor rozeznávající vzory
qPCR	quantitative polymerase chain reaction	kvantitativní polymerázová řetězová reakce
rcDNA	relaxed circular DNA	relaxovaná kruhová DNA

RIG-I	retinoic acid-inducible gene I	retinovou kyselinou indukovaný gen I
RLR	RIG-like receptor	RIG-podobný receptor
RR	regulatory receptors	regulační receptory
SARS-COV-2	severe acute respiratory syndrome coronavirus 2	koronavirus 2 způsobující těžký akutní respirační syndrom
SOCS-1	suppressor of cytokine signaling 1	supresor cytokinové signalizace 1
Src	Src kinase	kináza Src
STAT	signal transducer and activator of transcription	signální a aktivátorový protein
SVP	subviral particles	subvirové částice
Syk	protein kinase Syk	protein kináza Syk
TAB	TGF-beta-activated kinase 1 and MAP3K7-binding protein 1	kináza aktivovaná TGF-β a MAP3K7 vazebný protein
TAK-1	TGF-β activated kinase	kináza aktivovaná TGF-β
TCR	T cell receptor	receptor lymfocytů T
TfR	transferrin receptor	transferinový receptor
TGF-β	tumor growth factor β	tumor růstový faktor β
TIM-3	T-cell immunoglobulin and mucin-domain containing-3	protein 3 obsahující mucinovou doménu a imunoglobulin lymfocytů T
TIR	toll/IL-1 receptor	toll/IL-1 receptor
TLR	toll-like receptor	receptor podobný Toll
TNF-α	tumor necrosis factor α	tumor nekrotizující faktor α
TRAF	TNF receptor associated factor	faktor asociovaný s receptorem pro TNF-α
TRAIL	TNF-related apoptosis-inducing ligand	ligand indukující apoptózu spojený s TNF
TRAIL-R	TRAIL receptor	receptor pro TRAIL
TREM	triggering receptor expressed on myeloid cells-1	aktivační receptor myeloidních buněk
TRIF	TIR domain-containing adapter protein inducing IFN-Beta	adaptorový protein obsahující doménu TIR indukující produkci IFN-β
TRIM	tripartite motif-containing protein	protein obsahující tripartitní motiv
TYK	tyrosine kinase	tyrosinová kináza
UDG	uracil DNA glycosylase	uracil dna glykosyláza
UNC93B1	unc-93 homolog B1	Unc-93 homolog B1

VAMP3	vesicle-associated membrane protein 3	vezikulární membránový protein 3
Vav1	Vav1 protooncogene	protoonkogen Vav 1
Vpu	viral protein u	virový protein u

Abstrakt

Plasmacytoidní dendritické buňky (pDC) představují populaci imunitních buněk specializovanou na rozeznání virových nukleových kyselin pomocí endosomálních Toll-like receptorů 7 (TLR7) a 9 (TLR9). Aktivace TLR7/9 u pDC vede k produkci interferonů typu I (IFN-I). pDC dále přispívají k antivirové odpovědi prezentací virových antigenů lymfocytům T, čímž tvoří spojení mezi vrozenou a adaptivní složkou imunitní odpovědi.

Jelikož je nadměrná produkce IFN-I u pDC asociována s rozvojem autoimunitních onemocnění, musí být jejich aktivita přísně regulována. Z toho důvodu mají pDC na svém povrchu mnoho regulačních receptorů (RR), které inhibují TLR7/9. Tématem této disertační práce je objasnění mechanismu funkce RR u pDC a studie interakcí mezi pDC a obalenými viry schopných využít RR: viru hepatitidy B (HBV) a viru lidské imunodeficience (HIV). Naše výsledky prokázaly, že signální dráha MEK-ERK inhibuje produkci IFN-I u pDC a představuje tak důležitý element signalizace RR. Studie provedené naší výzkumnou skupinou naznačují, že farmakologické cílení signální dráhy MEK1/2-ERK u pDC může představovat jednu ze strategií obnovení funkčního fenotypu pDC. Dále jsme analyzovali, zda aktivní retrovirové terapie (ART) u skupiny 21 pacientů s chronickou infekcí HIV má vliv na množství a fenotyp pDC. Zjistili jsme, že chronická infekce HIV indukuje u pDC stav tzv. vyčerpaného fenotypu, který je asociován se zvýšenou expresí povrchových molekul TIM-3 a TRAIL. ART vedla u pacientů s chronickou infekcí HIV pouze k částečnému obnovení frekvence a fenotypu pDC. Naše výsledky dále naznačily, že množství povrchového TIM-3 u pDC lze využít jako prediktivní znak poklesu virové nálože HIV během ART. V případě HBV jsme zjistili, že pDC aktivované pomocí různých syntetických agonistů TLR7/9 účinně inhibují replikaci HBV v buněčných hepatomových liniích i v primárních lidských hepatocytech. Nicméně, naše výsledky dále prokázaly, že cílení různých imunitních buněk, nejen pDC, pomocí syntetických agonistů TLR, může představovat efektivnější možnost cílené terapie v případě chronické infekce HBV.

Tato disertační práce předkládá a diskutuje originální výsledky získané studiem mechanismu funkce vlivu RR na signalizaci TLR7/9 u pDC a efekt pDC aktivovaných pomocí syntetických agonistů TLR7/9 na životní cyklus HBV.

Klíčová slova: pDC, HBV, HIV, regulační receptory, interferon

Abstract

Plasmacytoid dendritic cells (pDC) are a highly specialized subset of immune cells that sense viral nucleic acids by endosomal toll-like receptors 7 and 9 (TLR7/9). Activation of TLR7/9 leads to the production of type I interferons (IFN-I). Moreover, pDC contribute to the antiviral response by presenting viral antigens to T lymphocytes and link innate and adaptive immunity.

pDC need to be properly regulated in order to limit excessive production of IFN-I that is associated with autoimmune diseases. Therefore, pDC possess a battery of regulatory receptors (RR) that limit TLR7/9-mediated cytokine production. This thesis focuses on the mechanism of RR-mediated inhibition of IFN-I production in pDC and explores interactions between pDC and two enveloped viruses, that possess the ability to hijack RR in pDC: hepatitis B virus (HBV) and human immunodeficiency virus (HIV). We showed, that MEK-ERK signaling pathway plays an active role in RR-mediated inhibition of IFN-I in pDC. Our results indicate that in line with other studies of our group, pharmacological targeting of MEK1/2-ERK signaling could be a strategy to re-establish immunogenic activity of pDC. Then, we investigated whether antiretroviral therapy (ART) in a cohort of 21 treatment-naive chronic HIV-infected patients has restored the number and phenotype of pDC. We found that chronic HIV infection induces exhausted phenotype of pDC that is associated with the elevated expression of TIM-3 and TRAIL. The pDC frequency and phenotype was only partially restored after ART. Moreover, our results suggested that the level of TIM-3 could be used as a predictive marker of HIV RNA decline during ART. Finally, we demonstrated that pDC activated with various TLR7/9 synthetic ligands inhibit HBV replication in hepatoma cell lines and primary human hepatocytes. Our results indicated that targeting of various TLRs in other immune cells and not specifically pDC, could be a better strategy towards an HBV cure.

Collectively, this thesis presents and assesses novel results that uncover the interplay between positive and negative signals in pDC triggered by TLR7/9 and RR, and the effect of TLR7/9-activated pDC on the HBV lifecycle.

Key words: pDC, HBV, HIV, regulatory receptors, interferon

1. Úvod

Lidský imunitní systém je vystaven nespočetnému množství patogenů, mezi které patří i mnoho různých virů. Zatímco některé viry nás infikují pouze dočasně a povětšinou způsobují lehká onemocnění, jiné viry mají schopnost perzistovat v našem těle celoživotně. Některé viry jsou schopné svým působením způsobit i rakovinové bujení, přestože na první pohled je prvotní infekce mírná a bez vážnějších patologických změn. Studium vztahu virů a imunitního systému ještě podtrhla celosvětová pandemie vyvolaná novým typem koronaviru SARS-CoV-2. Tato pandemie poukázala na skutečnost, že viry představují pro globalizovanou společnost neviditelného vetřelce, proti kterému se jen velmi těžko bojuje. Schopnost rychlé reakce ze strany vědecké komunity na nový virus je proto klíčová pro zmírnění dopadů na společnost.

Jednou z buněčných populací, o které bude tato disertační práce pojednávat, jsou plasmacytoidní dendritické buňky (pDC). Vzhledem ke svým vlastnostem, pDC představují důležitou zbraň imunitního systému vyvinutou pro rychlé rozeznání a reakci proti virové infekci. Aktivita pDC musí být v rámci imunitního systému přísně regulována, neboť jejich neadekvátní odpověď se podílí na vzniku mnoha autoimunitních onemocnění. Z toho důvodu jsou pDC vybaveny širokou paletou membránových receptorů, které regulují jejich aktivitu. Objasnění funkce receptorů schopných regulovat aktivitu pDC a také schopnost různých virů využívat tyto receptory za účelem úniku před pDC je jedním z témat této disertační práce.

Tato disertační práce se dále zabývá schopností pDC inhibovat replikaci viru hepatitidy typu B (HBV). I přes dostupnost očkování představuje HBV nadále celosvětový medicínský problém, neboť neexistuje efektivní léčba, která by vedla k vyléčení pacientů s chronickou infekcí HBV. Současným tématem výzkumu HBV je i možnost využití imunoterapie u pacientů s HBV. Studium vztahu pDC a HBV je proto klíčové, neboť pDC díky svým vlastnostem představují jeden z možných cílů terapie.

2. Literární přehled

2.1 Obecná charakteristika pDC

pDC představují důležitou populaci buněk vrozeného imunitního systému podílejících se na obraně hostitele proti virové infekci. Historicky byly toto buňky pojmenovány jako interferon-produkující buňky, neboť po vystavení viru sekretovaly značné množství interferonu typu I (IFN-I) (Fitzgerald-Bocarsly, 1993). Vyjma IFN-I jsou ale pDC schopné produkovat také interferony typu III (IFN-III) (Yin et al., 2012), které mají specifickou antivirovou funkci na epiteliích (Syedbasha and Egli, 2017). Po vystavení pDC viru nedochází pouze k sekreci IFN-I, ale také k morfologickým změnám, kdy se hladce kulatý tvar buněk rychle mění na buňky s typickými dendrity (obrázek 1) (Siegal et al., 1999). Z tohoto důvodu byly pDC zařazeny mezi dendritické buňky, které byly dále rozděleny na tzv. myeloidní (konvenční) dendritické buňky mDC a pDC (O'Doherty et al., 1994). Toto rozdělení je prováděno zejména na základě povrchových molekul lokalizovaných na povrchu pDC a mDC. Rozvoj nových metod jako RNA sekvenování jednotlivých buněk ovšem odhalilo, že rozdělení dendritických buněk na dvě hlavní linie je nedostatečné a jednotlivé subpopulace lze najít i v rámci pDC (Alculumbre et al., 2018). Jak již plyne z názvu pDC, tyto buňky jsou schopné vyjma rozeznání virové infekce také prezentovat na svém povrchu virové antigeny v komplexu s MHC glykoproteiny II. třídy a plnit tak roli profesionálních buněk prezentujících antigen. pDC tak tvoří důležité pojítko mezi vrozenou a adaptivní imunitou (Kadowaki et al., 2000). Kromě aktivace adaptivní odpovědi byla popsána i role pDC v indukci tolerance (Matta et al., 2010). pDC exprimují ligand inducibilního kostimulátoru lymfocytů T (ICOS-L) a podílejí se na indukci regulačních lymfocytů T (Faget et al., 2012). pDC tak mohou aktivovat i inhibovat adaptivní imunitní odpověď v závislosti na typu daného stimulu a plnit variabilní úlohu v rámci regulace imunitního systému. Velmi variabilní je také úloha pDC v případě nádorového mikroprostředí. Zatímco v případě rakovin žaludku a štítné žlázy je přítomnost pDC v nádorovém mikroprostředí negativním prognostickým faktorem vyplývající patrně z imunotolerantní role pDC v těchto nádorech (Liu et al., 2019; Yu et al., 2013), u nádorů prsu a slinivky představují pDC pozitivní ukazatel přežití pacientů (Kini Bailur et al., 2016; Tjomsland et al., 2010). Zejména díky pozitivnímu působení proti rakovinnému bujení se pDC staly předmětem cílené imunoterapie nádorů (Drobits et al., 2012). V neposlední řadě jsou pDC asociovány s mnoha autoimunitními onemocněními, jako je systémový lupus erythematodes a další (Li et al., 2017). pDC hrají různorodou úlohu v rámci imunitního

systému a jejich funkce musí být přísně regulována. Tato práce se vzhledem ke své charakteristice bude věnovat zejména schopnosti pDC rozeznat virovou infekci a mechanismy vrozené imunity působícími proti virové infekce v infikovaných buňkách. Dále bude práce pojednávat o mechanismech virů cílících regulační dráhy pDC za účelem utlumení antivirového působení pDC.

Obrázek 1: **Maturace pDC.** Srovnání nematurované pDC (vlevo) a maturované pDC maturované pomocí syntetického agonisty Toll-like receptoru 9 (TLR9) CpG-A (vpravo). Obrázek převzat a upraven podle (Bauer et al., 2001).

2.2 Základní povrchové markery pDC, možnosti izolace z periferní krve a dostupné buněčné linie

Frekvence pDC v periferní krvi se pohybuje od 0,2-0,8 % a jedná se tedy o nepočetnou populaci. Mezi základní povrchové znaky pDC patří CD123, CD4, CD45RA, lidský leukocytární antigen DR (HLA-DR), krevní antigen dendritických buněk 2 a 4 (BDCA-2, BDCA-4), a protein podobný transkriptu imunoglobulinů 7 (ILT-7), naopak molekuly typické pro další krevní buněčné populace jako CD11c, CD16 a další se na povrchu pDC nevyskytují (Liu, 2005). Pro izolaci z periferní krve se ve většině dostupné literatury setkáváme s využitím 2 postupů, a to izolace pomocí magnetických kuliček nebo pomocí FACS (Fluorescence activated cell sorting). Obě metody lze navíc vzájemně kombinovat (Wrzesinski et al., 2013). Při metodě FACS dochází k třídění pozitivních buněk pomocí specializovaného průtokového cytometru, přičemž lze využít specifické markery pDC (Bendriss-Vermare et al., 2005). Mnohem hojněji využívanou metodou je ovšem izolace pomocí magnetických kuliček, při které se využívá negativní nebo pozitivní selekce. V případě negativní selekce jsou magnetické kuličky obaleny protilátkami proti běžným znakům imunitních buněk, které nejsou přítomny na povrchu pDC. pDC tedy

nejsou označené žádnou protilátkou a neváží se na magnetické kuličky. Pozitivní selekce využívá naopak povrchové znaky přítomné na povrchu pDC jako je BDCA-4 a další (Dzionek et al., 2001). Nejnovější komerční izolační kity pro pDC jsou založené na dvoukrokové izolaci zahrnující negativní selekci následovanou pozitivní selekcí, přičemž čistota izolovaných pDC se pohybuje kolem 75-95 % oproti jednokrokové negativní selekci, kdy se čistota pohybuje kolem 50-70 % (Gondois-Rey et al., 2009). Nevýhoda pozitivní selekce může spočívat v tom, že některé specifické znaky pDC patří do rodiny tzv. regulačních receptorů a pozitivní selekce tedy může vést k částečné aktivaci těchto receptorů u pDC (Fanning et al., 2006). Pro studium regulačních signalizačních drah pDC je proto vhodnější využít negativní selekci, přestože čistota izolovaných pDC je nižší. Regulační receptory budou podrobněji popsány v následujících kapitolách. pDC je možné kromě periferní krve izolovat i z tkání a pupečníkové krve novorozenců s využitím popsaných metodik (Breitling et al., 2006; Vangeti et al., 2019).

Další nevýhodou pDC je jejich krátká životaschopnost *in vitro*, která se u izolovaných pDC pohybuje kolem 60 % po 24 hodinách kultivace *in vitro* a dramaticky klesá na zhruba 30 % po 48 hodinách kultivace. Pro zvýšení životaschopnosti pDC je do kultivačního média přidáván rekombinantní interleukin 3 (IL-3), který zvyšuje životaschopnost na 90 % po 24 hodinách kultivace *in vitro* (Gibson et al., 2002). Pro práci *in vitro* je možné využít i několik dostupných buněčných linií, které vykazují charakteristiky pDC. Jedna z prvních buněčných linií pDC byla získána z periferní krve leukemického pacienta (Chaperot et al., 2001) a následně byla buněčnou kultivací *in vitro* vyselektována buněčná linie s označením GEN2.2 (Chaperot et al., 2006). Podobným způsobem byla připravena také buněčná linie CAL-1 (Maeda et al., 2005). Porovnáním s primárními pDC bylo následně zjištěno, že buněčné linie GEN2.2 a CAL-1 vykazují velmi podobnou genovou expresi a odpověď na stimulaci TLR9 jako pDC. Oproti primárním pDC vykazují buňky GEN2.2 i CAL-1 značný proliferační potenciál *in vitro* a některé specifické signální dráhy přítomné v primárních pDC nejsou v GEN2.2 ani v CAL-1 liniích aktivovány (Carmona-Sáez et al., 2017). Vždy je tedy nutné výsledky získané z buněčných linií GEN2.2 i CAL-1 ověřit na primárních pDC. Méně využívanou leukemickou buněčnou linií vykazující charakteristiky pDC je buněčná linie PMDC05 (Narita et al., 2009). V poslední době je také vyvíjena metoda diferenciace primárních pDC z krevních kmenových buněk *in vitro* (Díaz-Rodríguez et al., 2017).

2.3 pDC a receptory vrozené imunity

Vrozený imunitní systém je schopen rozpoznat virovou infekci pomocí tzv.„pattern-recognition receptorů“ (PRR), které jsou schopné rozpoznat molekulární vzory asociované s patogeny (PAMP). Na základě homologie domén se PRR dělí na 5 rodin: Toll-like receptory (TLR), RIG-I-like receptory (RLR), NOD-like receptory (NLR), lektinové receptory typu C (CLR) a AIM2-like receptory (ALR). Aktivace buněk pomocí PRR vede jak ke změně transkripce vedoucí například k produkci cytokinů a dalších imunomodulačních proteinů, tak i k netranskripční odpovědi buňky, jako je například fagocytóza (Brubaker et al., 2015). Vyjma PAMP jsou PRR schopné rozeznat i hostitelské molekuly asociované s poškozením tkáně (DAMP) (Gong et al., 2020). Replikace virů v infikovaných tkáních může vést k uvolňování DAMP, například při nekrotické smrti infikované buňky (Dapat et al., 2017; Tsai et al., 2014). Výsledná imunitní odpověď je tak vždy složena z výstupů signálů PAMP a DAMP, čímž je zajištěna adekvátní odpověď imunitního systému.

pDC využívají pro rozeznání virů zejména receptory ze skupiny TLR. Receptorová rodina TLR zahrnuje u člověka 10 receptorů (Kawasaki and Kawai, 2014). Sekvenčně homologní proteiny TLR byly objeveny u *Drosophila melanogaster*, kde se kromě antifungální imunity podílejí také na dorzo-ventrálním vývoji embrya (Lemaitre et al., 1996). Jednotlivé TLR rozeznávají unikátní struktury jako jsou bakteriální a kvasinkové složky buněčných stěn, různé formy ribonukleových (RNA) a deoxyribonukleových kyselin (DNA) a mnoho dalších (tabulka č.1). pDC exprimují oproti jiným buněčným typům značné množství endosomálního TLR7 a TLR9 (Hornung et al., 2002). TLR7 rozeznává jednořetězcovou RNA (Diebold et al., 2004) a TLR9 rozeznává dvojřetězcovou DNA bohatou na nemethylované CpG sekvence (Hemmi et al., 2000). pDC jsou tak plně vybaveny receptory pro rozeznání nukleových kyselin RNA i DNA virů. Kromě toho pDC slabě exprimují ještě TLR1, TLR10 a TLR6, jejichž funkce není v případě pDC plně objasněna (Hornung et al., 2002). U pacientů trpících systémovou sklerodermií bylo zjištěno, že pDC exprimují také TLR8 (Kioon et al., 2018). Vyjma endosomálních TLR7/9 jsou pDC schopné rozeznat také dvojřetězcovou RNA pomocí cytoplasmatického RIG-I (retinovou kyselinou indukovaný gen I) receptoru (Bruni et al., 2015). Význam TLR7 a TLR9 v rozeznání virových nukleových kyselin byl mnohokrát potvrzen na myším modelu, ve kterém myši deficientní v TLR7/9 vykazovaly vyšší virové titry zejména v případě

infekcí herpetickými viry (Bussey et al., 2019; Zucchini et al., 2008). pDC tak představují důležitou populaci buněk vrozené imunity specializující se na rozeznání virových infekcí.

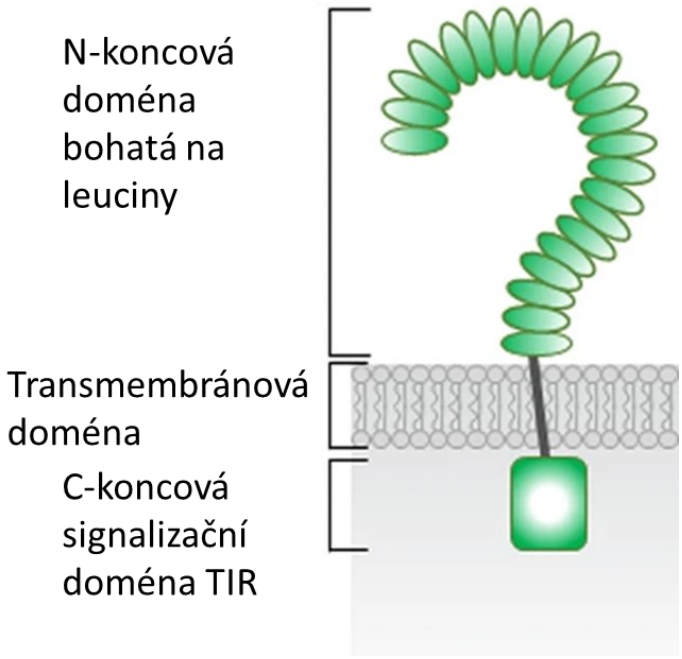
Tabulka č.1 – **Obecný popis lidských TLR a jejich ligandů.** Převzato a upraveno podle Frazão et al., 2013.

Receptor	Ligand	Signalizační adaptorový protein	Lokalizace Receptoru	Exprese receptoru
TLR1	triacilované lipopeptidy	MyD88	bunečný povrch	monocyty, makrofágy, lymfocyty B, myeloidní dendritické buňky
TLR2	Glykolipidy, HSP70, Zymosan	MyD88	buněčný povrch	monocyty, myeloidní dendritické buňky, žírné buňky
TLR3	dvojřetězcová RNA	TRIF	endosomy	myeloidní dendritické buňky, lymfocyty B
TLR4	LPS, HSP, fibrinogen, fragmenty heparan sulfátu a hyaluronové kyseliny	MyD88/TRIF	buněčný povrch	monocyty, makrofágy, myeloidní dendritické buňky, žírné buňky
TLR5	flagelin	MyD88	buněčný povrch	monocyty, makrofágy, myeloidní dendritické buňky,
TLR6	mnohonásobně diacylované lipopetidy	MyD88	buněčný povrch	monocyty, makrofágy, žírné buňky, lymfocyty B
TLR7	jednořetězcová RNA	MyD88	endosomy	plasmacytoidní dendritické buňky, monocyty, makrofágy, lymfocyty B
TLR8	jednořetězcová RNA	MyD88	endosomy	monocyty, makrofágy, žírné buňky
TLR9	DNA obsahující nemethylované dinukleotidy CpG	MyD88	endosomy	plazmacytodiní dendritické buňky, lymfocyty B, monocyty
TLR10	diacylované lipopetidy (predikce), HIV-gp41	MyD88	buněčný povrch	monocyty, makrofágy, lymfocyty B

MyD88=myeloidní diferenciační faktor 88, HSP70=protein teplotního šoku 70, TRIF=adaptorový protein obsahující doménu TIR a indukující produkci interferonu beta, LPS=lipopolysacharid, HIV-gp41= obalový protein gp41 viru lidské imunodeficience

2.3.1 Signalizace TLR7/9 u pDC

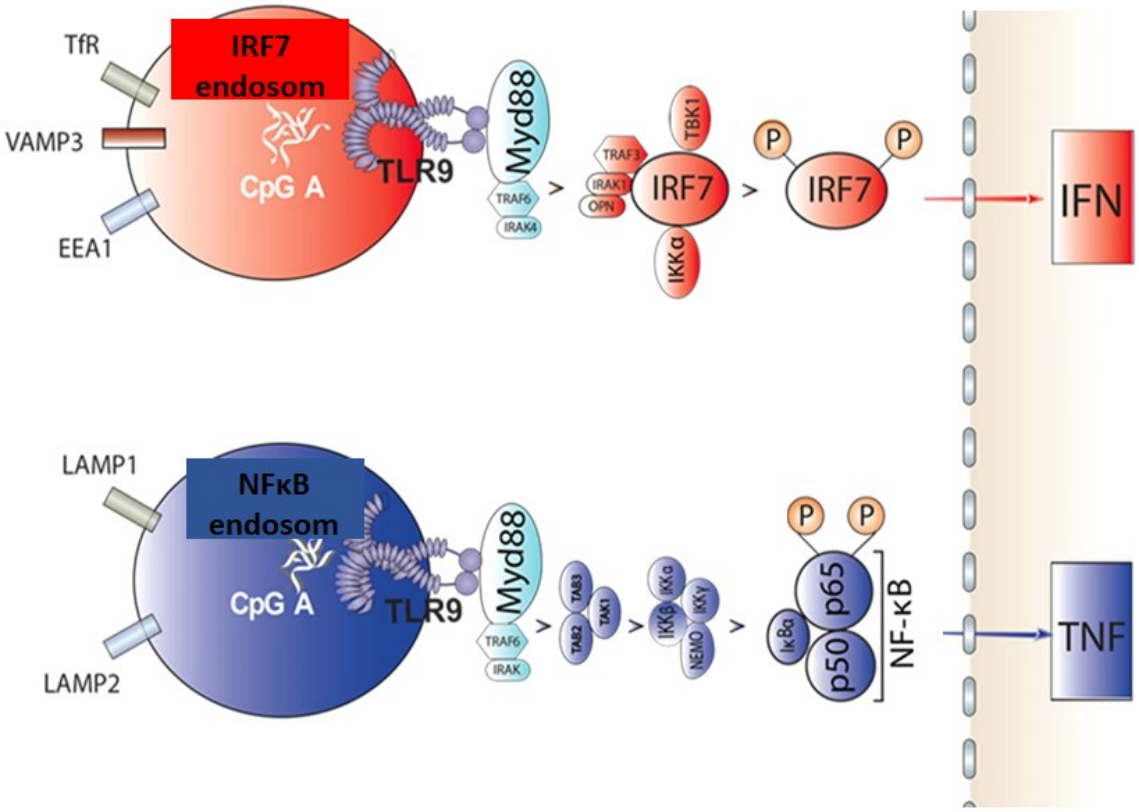
TLR7 i TLR9 mají podobnou strukturu. Jedná se o transmembránové receptory, jejichž N-koncová doména (ektodoména) obsahuje opakující se motivy bohaté na leucin, zajišťující vazbu ligandu, a cytoplasmatickou C-koncovou Toll/IL-1 receptor homologní doménu (TIR) vázající signalizační proteiny (Botos et al., 2011) (obrázek 2). Po vazbě ligandu dochází k homodimerizaci TLR7/9 a zahájení signální kaskády (Ohto et al., 2018; Zhang et al., 2018). Ke studiu funkce TLR7/9 se využívá syntetických ligandů těchto receptorů. Mezi nejvýznamnější aktivátory TLR9 patří tzv. CpG-A oligodeoxynukleotidy (ODN) obsahující nemethylované CpG sekvence (Krug et al., 2001). V případě TLR7 jsou využívány zejména imidazolchinolinové sloučeniny (Hemmi et al., 2002). Další zástupci syntetických aktivátorů TLR7/9 budou podrobně probrány v následujících kapitolách. Za klidového stavu je v pDC značná část TLR7/9 lokalizována v endoplazmatickém retikulu (ER). Po přidání ligandu TLR9 k pDC dochází k redistribuci TLR9 do endolysozomálních kompartmentů, kde dochází k vazbě ligandu na TLR9 (Latz et al., 2004). K retenci v ER a následné translokaci do endolysozomálního systému dochází i v případě TLR7 (Fukui et al., 2009). Na maturaci a redistribuci TLR7/9 do endolysozomálních kompartmentů se účastní celá řada proteinů jako chaperony gp96 (Yang et al., 2007) a protein asociovaný s TLR4 (PRAT4A) (Takahashi et al., 2007). Důležitý transportní chaperon představuje UNC93B1 (unc-93 homolog B1), který se kromě transportu TLR7/9 z ER (Kim et al., 2008; Lee et al., 2013) podílí i na stabilizaci TLR7/9 (Pelka et al., 2018). Transport TLR7/9 probíhá přes sekretorickou dráhu zahrnující Golgiho aparát. Pro účinnou vazbu ligandu v endolysozomálním systému musí dojít k naštěpení ekto-domény TLR7/9. Tento mechanismus patrně zabraňuje rozeznání vlastní DNA uvolněné například z mrtvých buněk (Park et al., 2008). Signalizace podílející se na redistribuci TLR7/9 z ER do endolysozomálních kompartmentů ani receptory řídící endocytózu syntetických ligandů TLR7/9 nejsou ovšem doposud plně známy.



Obrázek 2: **Základní schéma TLR**. N-koncová doména (ektodoména) bohatá na leucinové repetice se podílí na rozeznání a vazbě ligandu. Cytoplasmatická doména obsahuje Toll/IL-1 receptor homologní (TIR) doménu podílející se na signalizaci. Převzato a upraveno podle Chen et al., 2019.

Redistribuce TLR7/TLR9 do endolysozomálního systému je spojena s formováním tzv. signalizačních endosomů, kdy jednotlivé signalizační komplexy a výsledná exprese cytokinů závisí na lokalizaci TLR7/9 v endosomálním systému a dochází mezi nimi patrně k dynamickému přechodu (Combes et al., 2017). Po vazbě TLR7/9 ligandu na ektodoménu dochází k dimerizaci TLR7/9 a vazbě myeloidního diferenciačního faktoru 88 (MyD88) na TIR doménu TLR7/9. MyD88 dále váže faktor asociovaný s receptorem pro tumor nekrotizující faktor alfa (TNF-α) 3 a 6 (TRAF3/6) a kinázy asociované s receptorem pro interleukin-1 1 a 4 (IRAK1/4) nutné pro zahájení signalizace (Kawasaki and Kawai, 2014). Jestliže dojde k rozeznání ligandu pomocí TLR7/9 v časném endosomu a zároveň je schopen ligand indukovat retenci TLR7/9 v časném endosomu, dochází k asociaci s interferon regulačním faktorem 7 (IRF7) a vytváří se IRF7 signalizační endosom (Combes et al., 2017; Hirsch et al., 2017). IRF7 je v pDC na rozdíl od jiných buněčných typů konstitutivně exprimován (Kerkmann et al., 2003). IRF7 je následně fosforylován kinázami IKK-α/β (inhibitor of nuclear factor kappa-B kinase subunit alpha/beta) (Pauls et al., 2012) a TBK1 (TANK-binding kinase 1) (tenOever et al., 2004), translokuje se do jádra, kde se

podílí na indukci transkripce IFN-I/III (Gough et al., 2012). Pokud ligand TLR7/9 není schopen indukovat retenci TLR7/9 v časných endosomech, nastává rychlý přechod do lysozomů, kde se do komplexu TLR9/MyD88 váže IRF5 namísto IRF7. Dále dochází k aktivaci IKK-β pomocí kinázy TAK-1 (tumor growth factor β activated kinase) a aktivaci nukleárního faktoru κB (NF-κB), podle kterého nese tento endosom označení. V případě NF-κB endosomu dochází k produkci prozánětlivých cytokinů jako je TNF-α. Poměr a dynamika formování IRF7 versus NF-κB endosomů pak určí množství produkce IFN-I/III vůči prozánětlivým cytokinům (Combes et al., 2017; Hirsch et al., 2017) (obrázek 3). Bylo popsáno několik málo molekul nezbytných pro formování IRF7 endosomu jako PACSIN1 (protein kinase C and casein kinase in neurons 1) regulující aktinový cytoskelet (Esashi et al., 2012), jiné molekuly naopak podporují tvorbu NF-κB endosomu, jako je BAD-LAMP (brain and DC-associated LAMP-like molecule) (Combes et al., 2017). Přesný popis funkce a přechod mezi signalizačními endosomy u pDC ovšem vyžaduje další studium.



Obrázek 3. **Schéma signalizačních endosomů**. Vazba CpG-A na TLR9 v časném endosomu vede k formování IRF7 signalizačního endosomu, ve kterém dochází k fosforylaci transkripčního faktoru IRF7 pomocí TBK1 a IKK-α. Fosforylovaný IRF7 se podílí na transkripci IFN-I. Vazba CpG-A na TLR9 v lysozomu vede k fosforylaci transkripčního faktoru NF-κB a k produkci prozánětlivých cytokinů jako je TNF-α. Převzato a upraveno podle Combes et al., 2017. TfR=transferinový receptor, OPN=optineurin, IFN=interferony typu I a III, TNF=tumor nekrotizující faktor alfa.

Kromě hlavního signalizačního komplexu MyD88-IRF5/IRF7 se na TLR7/9 signalizaci v pDC a výsledné produkci cytokinů podílejí i další signální dráhy. Významnou úlohu hraje v pDC dráha PI3K-PKB/Akt-mTOR, která se podílí na indukci fosforylace IRF7 i NF-κB a tím i produkci IFN-I/III a prozánětlivých cytokinů (Cao et al., 2008; van de Laar et al., 2012). Další důležitou drahou regulující zejména produkci IFN-I/III je kináza p38 spadající do rodiny mitogenem aktivovaných proteinových kináz (MAPK) (Osawa et al., 2006). Naopak Brutonova tyrosinkináza (Btk) se podílí na pozitivní regulaci TLR9, ale nemá vliv na funkci TLR7 (Wang et al., 2014). Pro expresi IFN-I/IFN-III je zřejmě nutná

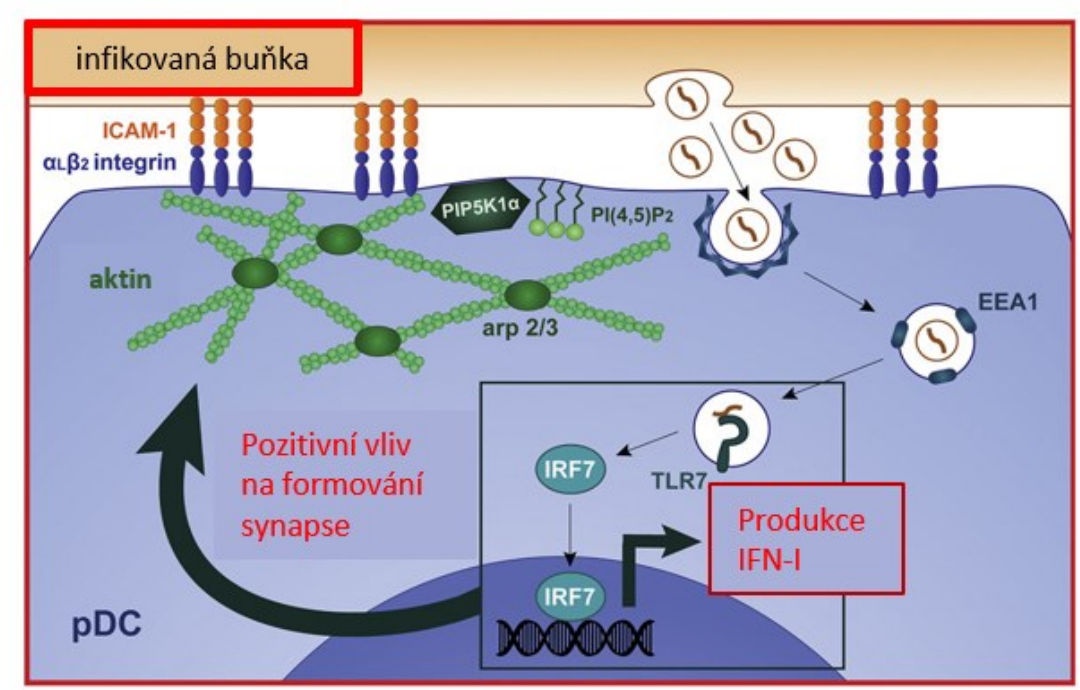
i remodelace chromatinu, neboť exprese IFN-I/III je u pDC silně blokována trichostatinem (Salvi et al., 2010). Další jaderné proteiny jako protein obsahující tripartitní motiv 8 (TRIM8) stabilizují IRF7 v jádře (Maarifi et al., 2019). V neposlední řadě je produkce IFN-I zesílena také kontaktem pDC s NK buňkami přes buněčné kontakty zahrnující LFA-1 (lymphocyte function-associated antigen 1) (Hagberg et al., 2011).

2.3.2 pDC a možnosti rozeznání virové infekce

pDC mají schopnost rozeznat jak volné virové částice, tak i virem infikované buňky. Nejjednodušší rozeznání virových částic je popsáno v případě velkých obalených DNA virů, jako jsou herpetické viry: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) nebo virus Epsteina-Barrové (EBV), kdy samotné virové částice jsou do pDC internalizovány a dochází k aktivaci TLR9 a produkci IFN-I/III bez nutnosti replikace těchto virů v pDC (Fiola et al., 2010; Lund et al., 2003; Siegal et al., 1999). Obdobným mechanismem dochází i k aktivaci TLR7 v případě rozeznání RNA virů jako je virus chřipky (Thomas et al., 2014). Role povrchových receptorů podílejících se na internalizaci virových částic do signalizačních endosomů v pDC není příliš objasněna. U viru hepatitidy typu A (HAV) bylo rozeznání virové RNA pomocí TLR7 inhibováno přídavkem rekombinantního annexinu V, což poukazuje na zapojení receptorů rozeznávající fosfatidylserin (Feng et al., 2015). V případě rozeznání viru HIV pDC byla popsána specifická interakce obalového proteinu gp120 a CD4 molekuly na povrchu pDC (Beignon et al., 2005). Na schopnost rozeznání virů u pDC má vliv také opsonizace virových částic protilátkami (Lannes et al., 2012; Veenhuis et al., 2017) a autofágie (Manuse et al., 2010).

Vystavení pDC virovým částicím obalených i neobalených virů nevede vždy k aktivaci pDC. V případě viru hepatitidy typu C (HCV) vede vystavení pDC virovým částicím naopak k inhibici TLR7/9 (Florentin et al., 2012). pDC jsou ovšem schopné rozeznat hepatocyty infikované HCV, neboť dochází k přenosu RNA HCV z infikovaných buněk do pDC pomocí exosomů. Pro aktivaci pDC je nicméně zapotřebí vysoká koncentrace exosomů nesoucích RNA HCV. Pro překonání aktivačního prahu TLR7/9 pomocí exosomů nesoucích RNA HCV je nutný těsný kontakt buňka-buňka, kdy dochází k polarizované sekreci exosomů z infikovaných buněk do pDC (Dreux et al., 2012). Assilová et al. nedávno popsala molekulární mechanismus formování kontaktu mezi pDC a infikovanými buňkami. pDC mají schopnost vytvářet s buňkami tranzientní kontakty

(<100 minut) skrze interakce mezi mezibuněčnými adhezivními molekulami (ICAM-1) a $\alpha_L\beta_2$ integriny a hrají tak roli aktivních strážců proti virem infikovaným buňkám. V případě, že dojde při tranzientním buněčném kontaktu k rozeznání virové RNA pomocí TLR7, dochází k reorganizaci buněčného kontaktu a vytvoření tzv. interferogenní synapse mezi infikovanou buňkou a pDC. Interferogenní synapse se vyznačuje reorganizací buněčného aktinu v místě buněčného kontaktu a vytvořením dlouhotrvajícího buněčného kontaktu (>350 minut) (obrázek 4). Tvorba interferogenní synapse je v případě pDC velmi konzervovaná, neboť autoři prokázali tvorbu synapsí nejen v případě HCV, ale i virů Dengue a Zika (Assil et al., 2019). Zdali dochází k tvorbě interferogenní synapse i v případě TLR9, není plně objasněno. U viru Dengue bylo navíc prokázáno, že nematurované částice nesoucí virovou RNA, ve kterých nedošlo k naštěpení prekurzorového membránového proteinu, jsou rozeznány v endosomálním systému TLR7. Maturované částice jsou naopak schopné před rozeznáním v endosomálním systému pDC uniknout, neboť dochází k fúzi virové částice s endosomem a transportu virového genomu do cytoplasmy (Décembre et al., 2014). Virus žluté zimnice má schopnost se v pDC replikovat a dochází k přímému rozeznání virových replikačních intermediátů pomocí cytoplasmatického RIG-I receptoru. Nicméně nepřímé rozeznání buněk infikovaných virem žluté zimnice pomocí TLR7 vede u pDC k mnohem silnější produkci IFN-I/III, což poukazuje na dominantní roli TLR7/9 u pDC v případě rozeznávání virových nukleových kyselin (Bruni et al., 2015). Rozeznání buněčným kontaktem se dále uplatňuje u viru lidské imunodeficience (HIV), kdy je opět účinnější, než v případě vystavení pDC virovým částicím HIV (Lepelley et al., 2011). Mnoho studií prokázalo schopnost pDC rozpoznat obalené RNA i DNA viry. Oproti tomu mechanismy rozeznání neobalených virů nejsou v literatuře tolik popsány. Výjimku tvoří rozeznání HAV, který je obecně považován za neobalený virus. HAV se šíří v hostiteli i tzv. obalenou formou (Feng et al., 2013) a právě tato obalená forma je silným aktivátorem produkce IFN-I u pDC, oproti isolovaným neobaleným kapsidám, které tvorbu IFN-I v pDC nestimulují. Zajímavostí je v případě HAV i fakt, že exosomy nesoucí RNA HAV neaktivují pDC (Feng et al., 2015).



Obrázek 4: **Interferogenní synapse.** Po aktivaci TLR7 pomocí virových PAMP v časných endosomech dochází k aktivaci IRF7. IRF7 indukuje produkci IFN-I. Signalizace TLR7 dále indukuje reorganizaci buněčného aktinu, čímž dochází k vytvoření dlouhotrvajícího buněčného kontaktu mezi infikovanou buňkou a pDC, který je závislý na interakci ICAM-I a integrinu αLβ2. Převzato a upraveno podle Assil et al., 2019. EEA1=antigen časného endosomu 1

2.3.3 Aktivace pDC pomocí syntetických ligandů TLR7/9

Rozvoj syntetických aktivátorů pDC započal se zjištěním, že krátké ODN obsahující nemethylované CpG sekvence jsou schopné vyvolat silnou produkci IFN-I po stimulaci krevních periferních mononukleárních buněk (PBMC) (Krug et al., 2001). Vzhledem k důležitosti IFN-I bylo jasné, že syntetičtí agonisté schopné aktivovat pDC mohou představovat nástroj pro léčbu různých onemocnění včetně virových infekcí. Od té doby došlo a dochází k vývoji a testování mnoha různých ligandů TLR. V současné době je intenzivně testován vliv tzv. duálních agonistů TLR, kdy fúzí dvou rozdílných ligandů TLR vzniká molekula schopná současně aktivovat větší množství TLR (Macedo et al., 2018). Vyjma vývoje nových ligandů TLR je dále testováno mnoho metod souvisejících s jejich biodistribucí *in vivo* a specifickým cílením do vybraných tkání (Madan-Lala et al.,

2017; Schau et al., 2019). Vzhledem k charakteru této práce bude výběr popsaných TLR ligandů omezený zejména s ohledem na jejich schopnost aktivovat pDC.

Jak již bylo zmíněno, TLR7 rozeznává jednořetězcovou RNA (Diebold et al., 2004). Krátké jednořetězcové oligoribonukleotidy (ORN) bohaté na polyU nebo GU sekvence jsou běžné aktivátory TLR7 *in vitro* (Lan et al., 2009). V případě stimulace TLR7 se nicméně častěji využívají syntetické analogy bazí a imidazolchinolinové sloučeniny. Vzhledem k sekvenční a funkční homologii mezi lidským TLR7 a TLR8, disponuje značná část syntetických ligandů TLR7 schopností aktivovat za určitých podmínek i TLR8 a fungovat tak jako duální agonisté (Patinote et al., 2020). Jeden z prvních imidazolchinolinů vykazující antivirové působení proti viru HSV-2 byl resiquimod (R848) (Bernstein et al., 2001). Na myším modelu bylo zjištěno, že antitumorogenní a antivirový potenciál R848 je asociován s aktivací TLR7 (Hemmi et al., 2002). Studie na lidských buňkách dále prokázala, že R848 je kromě TLR7 schopen aktivovat i TLR8 v závislosti na koncentraci (Jurk et al., 2002). R848 lze tedy za určitých podmínek považovat za duálního agonistu, neboť nezávisle aktivuje dva různé TLR. V dnešní době je již vyvinuto mnoho specifických TLR7 i TLR8 ligandů. Jedním z nich je například adeninový analog s označením CL264 vykazující selektivitu pouze k TLR7 (Hirota et al., 2002). Dalším aktivátorem TLR7 je malá syntetická molekula s označením GS-9620 (vesatolimod) vyvinutá firmou Gilead Sciences (Fosdick et al., 2014). Nespornou výhodou GS-9620 je možnost orálního podávání pacientům a vysoká tolerance léčby bez závažných vedlejších efektů (Agarwal et al., 2018). Poměrně novým agonistou TLR7 je molekula s označením CL413 (Adilipolin). CL413 vznikl kovalentním spojením 8-hydroxyadeninu a diacylovaného lipopeptidu Pam2CSK4. Analog báze představuje v případě CL413 syntetického agonistu TLR7 a diacylovaný peptid agonistu TLR2. CL413 představuje typického duálního agonistu TLR (Macedo et al., 2018). Z duálního charakteru syntetických ligandů vyplývá i jejich schopnost aktivovat mnohem širší spektrum imunitních buněk. Zatímco specifický syntetický ligand TLR7 jako je GS-960 a CL264 aktivují zejména pDC, od R848 a CL413 lze očekávat mnohem širší spektrum aktivace imunitních buněk.

Zlatým standardem pro aktivaci TLR9 pomocí syntetických ligandů představují krátké ODN obsahující nemethylované dinukleotidové CpG sekvence. Podle struktury a imunostimulačního efektu se ODN dále dělí do několika tříd. Mezi nejúčinnější induktory produkce IFN-I/III patří ODN třídy A (CpG-A). CpG-A charakterizuje přítomnost centrálního CpG motivu, 5´ koncového palindromu a přítomnost poly(G) motivu na 3´

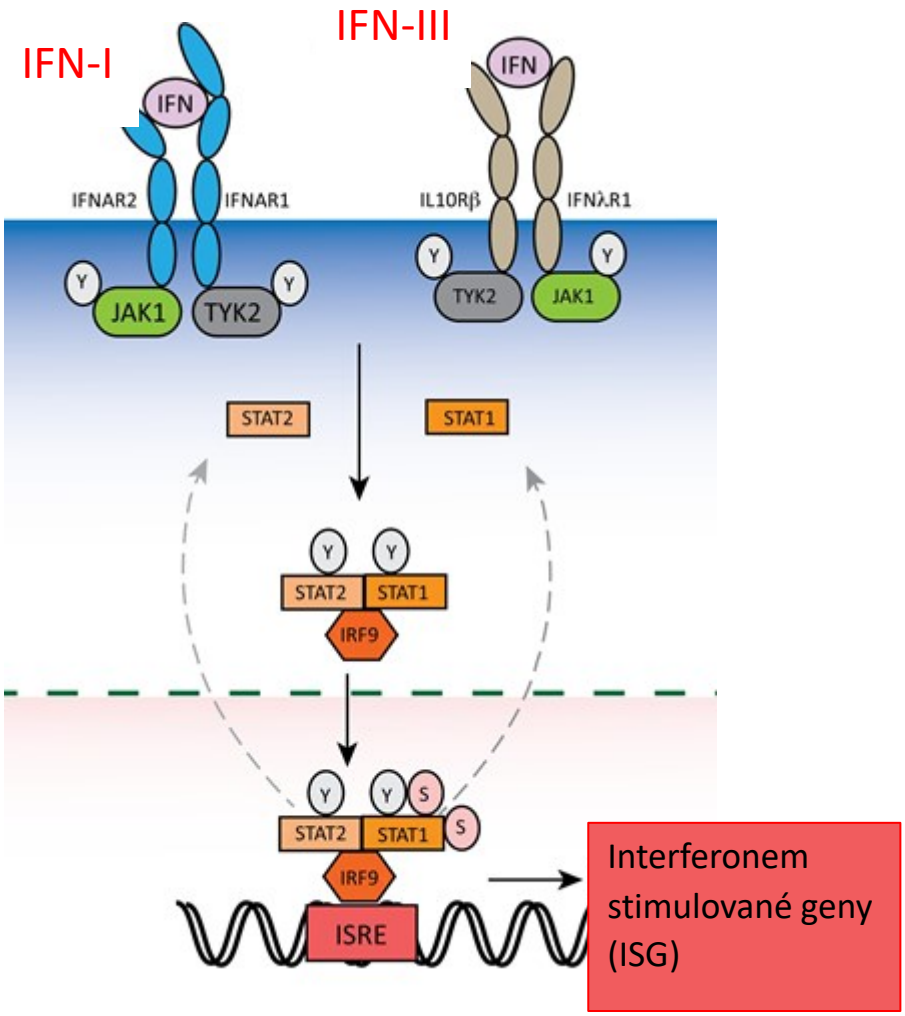
konci (Krug et al., 2001). Důvodem vysoké kapacity CpG-A na indukci IFN-I/III je patrně schopnost vytvářet struktury vyššího řádu (Klein et al., 2010), které zajišťují retenci CpG-A v časných endosomech (Combes et al., 2017) (kapitola 2.3.1). Struktury vyššího řádu vznikají v případě CpG-A díky interakcím poly(G) 3´ koncových motivů (Klein et al., 2010). Při aktivaci PBMC pomocí CpG-A dochází k aktivaci pouze pDC a predominatní odpovědí je produkce IFN-I/III. CpG-A vykazují tedy vysokou aktivační selektivitu vůči pDC (Hanagata, 2012). ODN třídy B (CpG-B) neobsahuje palindromy a poly(G) sekvence a vyvolává v pDC produkci zejména prozánětlivých cytokinů (Kerkmann et al., 2003). V případě aktivace PBMC pomocí CpG-B dochází k navíc k predominantní aktivaci B lymfocytů (Hanagata, 2012; Hartmann et al., 2000), které stejně jako pDC exprimují TLR9 (Jiang et al., 2007). Novějšími typy ODN jsou třídy C a P, které kombinují vlastnosti CpG-A a CpG-B a mají schopnost aktivovat pDC i lymfocyty B (Hanagata, 2012).

2.3.4 Vliv aktivovaných pDC na virem infikované buňky

pDC produkují 100 – 1000 krát více IFN-I než ostatní buněčné typy a představují tak významné producenty IFN-I v lidském těle (Liu, 2005). IFN-I jsou hlavní složkou vrozené imunity podílející se na inhibici virového šíření v časných fázích virové infekce. Vyjma IFN-I produkují pDC také mnoho dalších cytokinů a chemokinů, které se společně s IFN-I podílejí na antivirové odpovědi v místě infekce a na migraci imunitních buněk do místa infekce. pDC prezentací virových antigenů na MHC glykoproteinech II. třídy společně s expresí kostimulačních molekul CD80 a CD86 mohou následně aktivovat cytotoxické lymfocyty T (Kadowaki et al., 2000). Vzhledem k charakteru této práce, bude tato kapitola pojednávat zejména o mechanismech působení IFN-I/III na virovou replikaci.

Interferony se dělí podle struktury a receptorů do tří typů: Interferony typu I (IFN-I), Interferony typu II (IFN-II) a Interferony typu III (IFN-III). IFN-I zahrnující 13 subtypů IFN-α, IFN-β a několik dalších genových produktů (IFN-ε, IFN-τ, IFN-κ), jejichž funkce není příliš definována. Jediným zástupcem IFN-II je interferon gamma (IFN-γ). IFN-III zahrnují 4 subtypy tzv. interferonu lambda (IFN-λ) (McNab et al., 2015). pDC produkují IFN-I a IFN-III (Yin et al., 2012). Produkci IFN-γ ovšem mohou pDC vyvolat nepřímo působením IFN-α na NK buňky (Marshall et al., 2006). Interferony I/III indukují v místě virové infekce tzv. antivirový stav, a to jak v infikovaných, tak i v neinfikovaných buňkách a tím zabraňují šíření virové infekce. Antivirový stav je navozen vazbou IFN-I/III na

heterodimerní receptor lokalizovaný na buněčném povrchu. Zatímco receptory pro IFN-I (IFNAR) jsou široce exprimovány, exprese IFN-III receptorů (IFNLR) je omezena především na epiteliální buňky (McNab et al., 2015). Přestože IFN-I/III využívají rozdílné receptory, vazba na receptor vyvolá v obou případech aktivaci JAK/STAT signální dráhy, která spouští expresi interferonem stimulovaných genů (ISG) (obrázek 5). Mezi ISG patří mnoho proteinů podílejících se na restrikci virové infekce (Ivashkiv and Donlin, 2014). Funkce několika vybraných ISG bude podrobněji popsána v následujících kapitolách. Význam duální indukce ISG pomocí IFN-I/III je předmětem mnoha studií. Jeden z modelů předpokládá, že IFN-III hrají důležitou roli v případě lokalizace virové infekce na epiteliích, kdy IFN-III vykazují antivirovou aktivitu a zároveň se podílejí na utlumení silné prozánětlivé odpovědi a podporují tak homeostázi tkáně. V souladu s tímto modelem je IFNLR heterodimer tvořený specifickým řetězcem IFNLR1 a řetězcem specifickým pro IL-10 (IL-10Rβ) (Obrázek 5). Při systémovém rozšíření virové infekce mimo epiteliální tkáně nastupuje naopak IFN-I, který má mnohem silnější antivirový a prozánětlivý účinek a má tedy za úkol zabránit systémovému šíření viru i za cenu vyššího poškození tkáně (Andreakos et al., 2019). Kromě IFN-I/III byl antivirový efekt popsán i pro další cytokiny produkované pDC jako je TNF-α a IL-6 (Kuo et al., 2009; Palumbo et al., 2015; Seo and Webster, 2002).



Obrázek 5: Signalizace IFNI/III. IFN-I se vážou na IFNAR1/2 a dochází k fosforylaci signálního a aktivátorového proteinu 1 a 2 (STAT1/2) pomocí janusovo kinázy 1 (JAK1) a tyrosinové kinázy 2 (TYK2). IFN-III se vážou na IL10Rβ a IFNλR1 a podobně jako v případě IFN-I dochází k fosforylaci STAT1/2. Fosforylovaný STAT1/STAT2 se společně s interferon regulačním faktorem 9 (IRF9) vážou na interferon stimulovaný element (ISRE) a spouští expresi interferonem stimulovaných genů (ISG). Převzato a upraveno podle (Wang et al., 2017).

Po aktivaci TLR7/9 pomocí syntetických ligandů nebo po expozici virem chřipky a HIV exprimují pDC na svém povrchu TRAIL (TNF-related apoptosis-inducing ligand) (Chaperot et al., 2006; Hardy et al., 2007). TRAIL je transmembránový protein II. typu patřící do TNF rodiny receptorů. Dojde-li k vazbě TRAIL na receptory TRAIL-R1 (DR4) nebo TRAIL-R2 (DR5), dochází k trimerizaci receptorů a spuštění signální kaskády vedoucí k apoptotické smrti buňky zahrnující aktivaci kaspáz 8 a 10 (Ashkenazi and Dixit,

1998; Pan et al., 1997). Většina tkání ale není citlivá k apoptóze indukované TRAIL (Ashkenazi and Dixit, 1998; Gura, 1997). Senzitivita k apoptóze indukované TRAIL nicméně významně roste u virem infikovaných buněk nebo u transformovaných buněk (Wang and El-Deiry, 2003). Mnoho studií prokázalo přímé cytotoxické působení pDC exprimujících TRAIL proti virem infikovaným i transformovaným buňkám (Babu et al., 2009; van Dijk et al., 2013). Exprese TRAIL závisí nejen na TLR7/9 signalizaci, ale také na pozitivní smyčce zahrnující aktivaci IFNAR, a je tedy opět závislá na produkci IFN-I (Chaperot et al., 2006; Hardy et al., 2007).

pDC mají schopnost aktivovat buňky vrozeného imunitního systému a tím podpořit antivirové působení. Aktivované pDC sekrecí IFN-α aktivují například NK buňky. Aktivace NK buněk je navíc silně zesílena přímým kontaktem s pDC (Conry et al., 2009). Zdali je v případě buněčného kontaktu vytvořena podobná buněčná synapse jako v případě rozeznávání infikovaných buněk (interferogenní synapse, kapitola 2.3.1) není ovšem jasné.

2.4 Negativní regulace TLR7/9 u pDC

IFN-I/III hrají důležitou úlohu během časné fáze virové infekce, nicméně následně musí dojít k regulaci jejich produkce. Nadměrná produkce IFN-I/III vede k imunopatologiím, které se souhrnně označují jako interferonopatie (Rodero and Crow, 2016). Z toho důvodu je nutné regulovat aktivitu pDC a vybalancovat časově i místně produkci IFN-I/III během virové infekce. Po stimulaci TLR7/9 v pDC dochází ke spuštění signálních drah schopných negativně regulovat signalizaci TLR7/9, čímž je zajištěna optimální produkce IFN-I/III (Karrich et al., 2013; Marshall et al., 2007). Na regulaci signalizace TLR se podílí mnoho mechanismů zahrnující degradaci klíčových signalizačních komplexů pomocí K48-vázané ubikvitinace nebo naopak deubikvitinace K63-vázaných polyubikvitinových řetězců, které jsou nutné pro sestavení signalizačních komplexů. Na negativní regulaci TLR se také podílí alternativní sestřih, mechanismy transkripční regulace, mikro RNA (miRNA) a mnoho dalších (Kondo et al., 2012).

Jedním z dobře popsaných regulátorů v případě pDC je miRNA-146a. miRNA-146a je silně exprimována po aktivaci TLR7/9. Zvýšená exprese miRNA-146a následně snižuje produkci IFN-I, prozánětlivých cytokinů i kostimulačních molekul a indukuje apoptózu pDC (Karrich et al., 2013). Studie na monocytech prokázala schopnost miRNA-146a cílit IRAK-1 a TRAF6 (Taganov et al., 2006). Mezi negativní regulátory TLR7/9 patří

i zástupci ISG. Zástupci E3 ubikvitin ligáz z rodiny TRIM jmenovitě TRIM20, 22, 36 a 28 inhibují produkci IFN-I v pDC (Maarifi et al., 2019). Po aktivaci pDC dochází také ke snížení exprese BAD-LAMP proteinu, který se podílí na dynamickém přechodu mezi signalizačními endosomy (Combes et al., 2017). Také aktivace TLR7 vede k inhibici TLR9 signalizace a dostupná data poukazují na skutečnost, že TLR7 signalizace dominuje nad TLR9 (Marshall et al., 2007). Na myším modelu bylo zjištěno, že i samotná přítomnost IFN-I/III v séru vede k poklesu pDC (Swiecki et al., 2011). Množství pDC a s tím spojená produkce IFN-I/III může být regulována i cytotoxickými lymfocyty T, které indukují apoptózu pDC (Mossu et al., 2016).

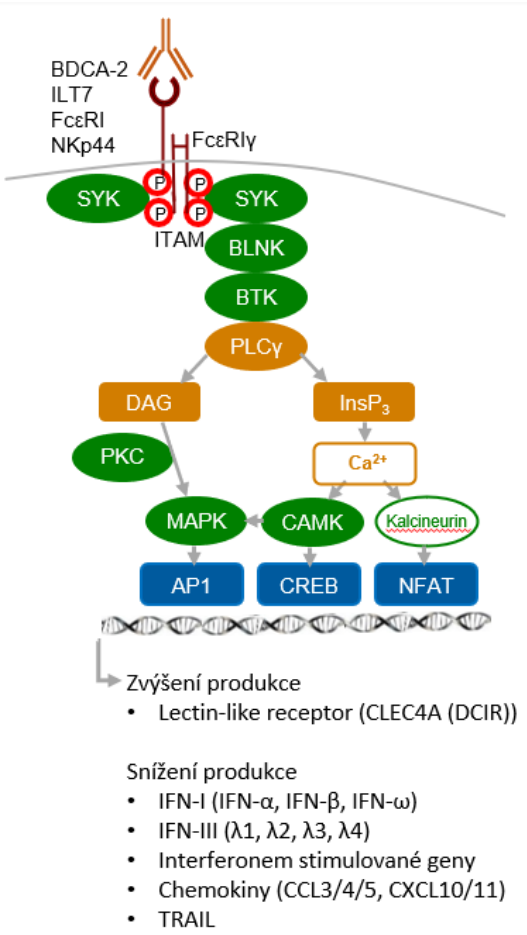
Skutečnost, že aktivace TLR7/9 v pDC vede k aktivaci zpětné negativní smyčky inhibující TLR7/9 byla nedávno podpořena také objevem molekuly TIM-3 (T-cell immunoglobulin and mucin-domain containing-3) na povrchu aktivovaných pDC (Schwartz et al., 2017). TIM-3 byl popsán již dříve jako molekula lokalizovaná na povrchu lymfocytů T u kterých plní roli inhibičního receptoru, kdy po vazbě ligandu tlumí aktivitu lymfocytů T a je asociován s jejich tzv. vyčerpáním (Wolf et al., 2020). Také přítomnost TIM-3 na povrchu pDC u pacientů s HIV je asociována s nižší produkcí IFN-α. IFN-α navíc zvyšuje expresi TIM-3 v pDC, což opět poukazuje na zapojení TIM-3 v negativní regulaci TLR7/9. Analýzou mechanismu funkce TIM-3 u pDC bylo zjištěno, že TIM-3 kolokalizuje s IRF7 a p85 podjednotkou fosfatidylinositol-3-kinázy (PI3K) v lysozomech a patrně interferuje s translokací IRF7 do jádra. Přesný mechanismus inhibiční funkce TIM-3 v pDC není ovšem plně vysvětlen. Aktivace signalizace TIM-3 pomocí cílených protilátek nevedla ke zvýšení inhibičního efektu na produkci IFN-I v pDC, což poukazuje na inhibiční roli TIM-3 nezávislou na vazbě ligandu (Schwartz et al., 2017). Nelze ovšem plně vyloučit, že vazbu ligandu a následná aktivace signalizace TIM3 ovlivňuje funkci pDC. pDC jsou kromě TIM-3 negativně regulovány velkým množstvím receptorů lokalizovaných na povrchu pDC.

2.4.1 Regulační receptory inhibující TLR7/9 v pDC

Regulační receptory (RR) představují různorodou skupinu plazmatických receptorů se schopností velmi účinně inhibovat produkci IFN-I/III a slouží tedy jako pojistka proti přílišné aktivaci pDC. Prvním popsaným regulačním receptorem u pDC byl BDCA-2. BDCA-2 představuje transmembránový protein vykazující zhruba 50% sekvenční homologii s myším Dectinem-2 a zhruba 40% sekvenční homologii s lidským imunoreceptorem dendritických buněk (DCIR) a patří do rodiny CLR. Analýzou PBMC

bylo zjištěno, že BDCA-2 je specificky exprimován v pDC a slouží k internalizaci antigenů do endosomálních kompartmentů procesujících antigeny (Dzionek et al., 2001). Několik dalších studií následně potvrdilo inhibiční vliv BDCA-2 na produkci IFN-I/III zprostředkovanou TLR7/9 u pDC (Cao et al., 2007; Jähn et al., 2010; Röck et al., 2007). Kromě produkce cytokinů ovlivňuje signalizace BDCA-2 také expresi kostimulačních molekul CD80 a CD86 v pDC (Jähn et al., 2010). BDCA-2 vykazuje afinitu vůči specifickým trisacharidům zakončených galaktózou, které se vyskytují na IgG, IgA a IgM protilátkách. Není ovšem jasné, zda vazba specifických trisacharidů na BDCA-2 vede ke spuštění signální dráhy nebo dochází pouze ke kompetici o vazbu na BDCA-2 (Jégouzo et al., 2015; Kim et al., 2018). Přesný význam vazby protilátek na BDCA-2 proto vyžaduje další studie. Dalším receptorem selektivně exprimovaným v pDC je ILT7 patřící do leukocytární imunoglobulin-like receptorové rodiny (LIR). ILT7 inhibuje podobně jako BDCA-2 produkci IFN-I/III a prozánětlivých cytokinů zprostředkovanou aktivací TLR7/9 u pDC. Nicméně oproti BDCA-2 neovlivňuje ILT7 expresi kostimulačních molekul CD80 a CD86 (Cao et al., 2006). ILT7 receptor specificky váže stromální antigen kostní dřeně 2 (BST-2) patřící mezi ISG. Interakce ILT7-BST2 opět ukazuje na negativní úlohu IFN-I/III v aktivaci TLR7/9 u pDC (Cao et al., 2009). Schopnost inhibice TLR7/9 v pDC byla prokázána i v případě receptoru NKp44 (natural killer cell P44-related protein) patřící do imunoglobulinové superrodiny. NKp44 je exprimován u tonsilárních pDC a u pDC kultivovaných *in vitro* v přítomnosti IL-3. Naopak krevní pDC nevykazovaly po izolaci expresi povrchového NKp44. NKp44 asociuje s adaptorovým proteinem DAP12 (DNAX adaptor protein 12) (Fuchs et al., 2005). Endogenními ligandy NKp44 jsou například PCNA (proliferating cell nuclear antigen) (Rosental et al., 2011), specifická sestřihová forma proteinu MLL-5 (mixed lineage leukemia 5) (Baychelier et al., 2013) a další. Novější studie odhalila přítomnost NKp44 také u pDC lokalizovaných v nádorovém mikroprostředí. Posledním popsaným RR u lidských pDC byl receptor leukocytů podobný imunoglobulinům 1 (LAIR-1) (Bonaccorsi et al., 2010). Atypickým zástupcem RR rodiny je DCIR, neboť přímo obsahuje inhibiční motiv imunoreceptorů založený na tyrosinu (ITIM) a neasociuje tak s adaptorovým proteinem jako ostatní popsané regulační receptory. Po aktivaci DCIR u pDC dochází k inhibici TLR7/9 dráhy (Meyer-Wentrup et al., 2008). Signalizace DCIR vykazuje podobnost se signalizací BDCA-2 a lze tedy předpokládat podobný mechanismus účinku na TLR7/9 dráhu u pDC (Florentin et al., 2012).

Signalizace BDCA-2 patří mezi nejvíce studovanou a popsanou mezi RR. pDC specificky exprimují 2 adaptorové proteiny nesoucí aktivační motiv imunoreceptorů založený na tyrosinu (ITAM) a to FcεRIγ (Fc fragment of IgE receptor Ig) a DAP12 (Cao et al., 2007). ITAM představuje velmi konzervovaný signální motiv se sekvencí YXXXL/I-X_{6-8}-YXXL/I (X představuje jakoukoliv AK). ITAM se uplatňuje v celé řadě signalizačních drah, jako jsou signalizace receptorů lymfocytů T (TCR), receptorů lymfocytů B (BCR) a mnoho dalších. Fosforylace tyrosinových zbytků v ITAM vede k vazbě mnoha kináz a zahájení signalizační kaskády (Underhill and Goodridge, 2007). BDCA-2 v pDC vytváří komplex s adaptorovým proteinem FcεRIγ (Cao et al., 2007; Röck et al., 2007). Po aktivaci BDCA-2 pomocí specifických protilátek dochází k fosforylaci kinázy Syk na Y525 a také kinázy Src na Y416. Dále je fosforylován adaptorový protein Slp65 (BLNK, B-cell linker)) a dochází k fosforylaci Vav1 a fosfolipázy Cγ2 (PLCγ2). Aktivovány jsou i extracelulárním signálem regulované kinázy 1 a 2 (ERK1/2) spadající do rodiny MAPK. Po aktivaci BDCA-2 dochází také k intracelulárnímu uvolnění vápníkových iontů (Ca^{2+}) (Cao et al., 2007; Dzionek et al., 2001). Inhibice kináz Syk a Src vede k zablokování jak vápníkové signalizace, tak i k fosforylaci Vav1 a PLCγ2. Syk a Src jsou tedy hlavní kinázy stojící na počátku BDCA-2 signální kaskády (Aouar et al., 2016). Adaptorový protein Slp65 by se mohl podílet na aktivaci PI3K, nicméně role Slp65 v signalizaci BDCA-2 není plně objasněna. Vzhledem k podobnostem signální dráhy BDCA-2 a dalších RR se signální drahou BCR bývá signalizace RR označována jako signalizace podobná BCR (BCR-like) (obrázek 6) (Cao et al., 2007). Studie Röckeho et al. naznačila, že součástí signalizace BDCA-2 je i kináza Lyn a Btk. Dále bylo zjištěno, že po aktivaci BDCA-2 dochází k rychlé internalizaci BDCA-2 do pozdního endosomu závislou na klatrinu. Studie potvrdila, že BDCA-2 vykazuje podobnosti se signalizací BCR, nicméně na rozdíl od signalizace BCR nedochází k aktivaci kanonické dráhy NF-κB, ale naopak k inhibici fosforylace inhibitoru NF-κB (IκBα) (Röck et al., 2007). Kináza Syk se neúčastní pouze signalizace BDCA-2, ale pozitivně reguluje také TLR7/9 (Aouar et al., 2016). Co se týče dalších receptorů z rodiny RR, ILT7 podobně jako BDCA-2 asociuje s adaptorovým proteinem FcεRIγ a lze očekávat, že i signalizace bude shodná s BDCA-2 (Cao et al., 2006). U dalších zástupců RR není signalizace v pDC v literatuře popsána, nicméně lze předpokládat podobný mechanismus jako u signalizace BDCA-2.



Obrázek 6. RR signalizace u pDC. Po aktivaci RR dochází k fosforylaci ITAM na adaptorovém proteinu FcεRIγ a následné vazbě kinázy Syk. Syk fosforyluje adaptorový protein BLNK a dochází k aktivaci fosfolipázy Cγ (PLCγ). Aktivace protein kinázy C (PKC) vede k aktivaci ERK1/2 (MAPK). Transientní zvýšení Ca^{2+} vede k aktivaci nukleárního faktoru aktivovaných lymfocytů T (NFAT) a k aktivaci kalmodulin kinázy (CAMK), která dále aktivuje transkripční faktor CREB (cAMP response element-binding protein). Finální efektem aktivace RR je snížení produkce IFN-I/IFN-III, ISG, TRAIL a chemokinů u pDC. Převzato a upraveno podle Hirsch et al., 2017. BTK=Brutonova tyrosin kináza.

2.4.2 Viry využívající funkci regulačních receptorů k inhibici produkce IFN-I/III v pDC

Mnoho virů vyvinulo strategie úniku před imunitním systémem hostitele. Také v případě rozeznání virové infekce pDC bylo popsáno několik mechanismů úniku virů. Modelový příklad v případě pDC byl popsán zejména pro HCV. Jak již bylo zmíněno, pDC rozeznávají exosomy obsahující RNA HCV, přičemž k dosažení optimálního přenosu aktivačního signálu je nutné vytvoření interferogenní synapse mezi pDC a buňkou infikovanou HCV (Assil et al., 2019). Florentin et al. však prokázali, že pokud jsou ve společné kultuře (kokultuře) pDC společně s hepatocyty, které produkují exosomy nesoucí RNA HCV, ponechány i HCV virové částice, nedochází k produkci IFN-I/III. Následnou analýzou bylo zjištěno, že HCV virové částice vážou BDCA-2 a DCIR a aktivují tak u pDC inhibiční signalizaci RR. Aktivační signál zahrnující exosomy nesoucí RNA HCV je tak plně inhibován inhibičním signálem představovaným virovými částicemi HCV. Výsledná

produkce IFN-I/III v pDC se proto řídí souhrou aktivačních a inhibičních signálů a jejich vzájemným působením (Florentin et al., 2012)

Podobná souhra aktivačních a inhibičních signálů modulujících funkci pDC byla popsána i pro HIV. pDC rozeznávají volné HIV partikule i HIV infikované buňky, přičemž rozeznání HIV infikovaných buněk je mnohem efektivnější (Lepelley et al., 2011). U pDC vystavených volným virovým částicím HIV dochází k aktivaci kinázy Syk. V porovnání s aktivací pDC virem chřipky, vede aktivace Syk zprostředkovaná HIV pravděpodobně k tzv. pozdní produkci IFN-I/III v pDC (Lo et al., 2012). Možným vysvětlením je patrně schopnost obalového glykoproteinu gp120 vázat BDCA-2 (Martinelli et al., 2007). Jiná práce ovšem nepotvrdila inhibiční vliv gp120 a naopak prokázala jeho aktivační vliv (Del Cornò et al., 2005). Jelikož jsou volné částice HIV velmi špatným induktorem produkce IFN-I/III v pDC, lze předpokládat, že virus HIV má schopnost aktivovat inhibiční dráhy TLR7/9 v pDC. HIV má schopnost inhibovat i rozeznání infikovaných buněk, které je závislé na buněčném kontaktu. HIV pomocí virového proteinu U (Vpu) reorganizuje na plasmatické membráně protein BST2 (alternativně nazývaný tetherin) (Bego et al., 2015). BST2 patří mezi ISG (Holmgren et al., 2015) a v případě HIV plní roli restrikčního faktoru. BST2 je schopný vázat virové částice HIV na plazmatické membráně a bránit tak uvolňování nových virových částic (Perez-Caballero et al., 2009). BST2 se vyskytuje na membráně ve dvou isoformách, přičemž obě jsou schopné vázat virové částice HIV. Vpu protein je schopen cílit jednu z isoforem BST2 k degradaci a dále je schopný druhou isoformu BST2 reorganizovat na plazmatické membráně. Membránový BST2 následně váže ILT7 receptor na povrchu pDC a inhibuje rozeznání HIV (Bego et al., 2015). Schopnost HIV modulovat funkci BST2 a tím i produkci IFN-I/III je závislá také na příslušném kmenu HIV (Bego et al., 2016). Dalším virem schopným vyvolat chronickou infekci ve svém hostiteli a modulovat funkci pDC je HBV. Vzhledem k tématu disertační práce bude vztah HBV a pDC popsán podrobně v následujících kapitolách.

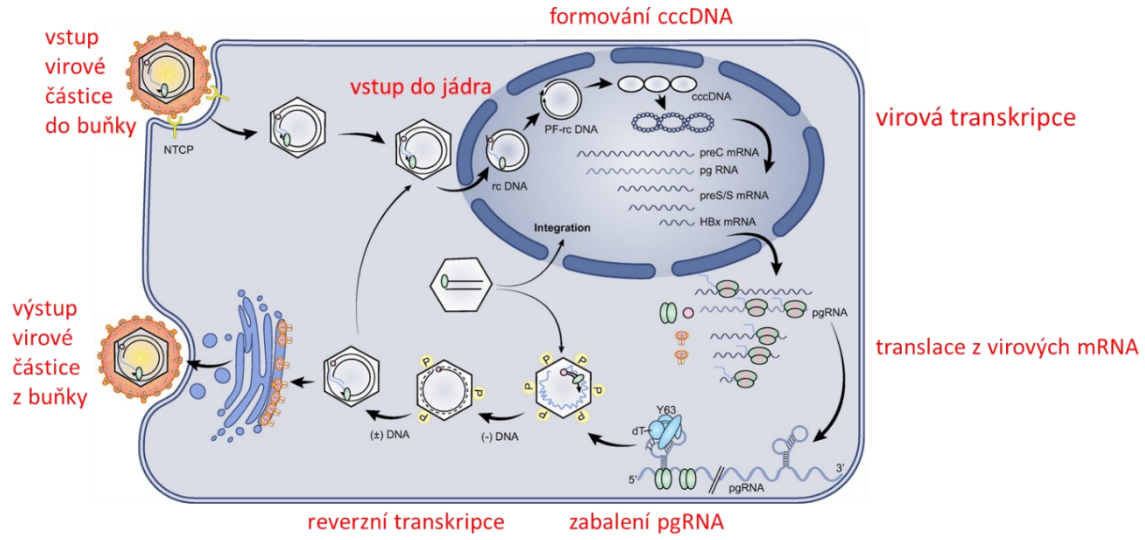
2.5. Základní charakteristika viru HBV

HBV je malý obalený DNA virus spadající do virové rodiny *Hepadnaviridae*. Virová rodina *Hepadnaviridae* zahrnuje skupinu hepatotropních virů schopných infikovat ptáky a savce, vyznačuje se úzkou hostitelskou specifitou a společným mechanismem replikace virového genomu (Lamontagne et al., 2016). Akutní infekce HBV přechází do chronické infekce u zhruba 5-10 % dospělých, zatímco u novorozenců dochází k přechodu

do chronické fáze u 90-95 %. V roce 2015 odhad počtu lidí chronicky infikovaných HBV činil přibližně 257 milionů. I přes dostupnost vakcíny představuje HBV značný medicínský problém vzhledem k rozvoji hepatocelulárního karcinomu u 25-40 % chronických nosičů HBV. Cílenou léčbu komplikuje také přítomnost 8 různých genotypů, jejichž genetická informace se liší i o více než 8 % (Seto et al., 2018).

HBV virová částice nese částečně dvouřetězcovou relaxovanou kruhovou DNA (rcDNA) o velikosti kolem 3,2 kpb. Na virovém genomu je kovalentně vázán P protein, který se podílí na replikaci virového genomu. Virový genom je obalen 120 dimery kapsidového proteinu. Virová kapsida dále nese lipidický obal obsahující 3 formy povrchového antigenu S (HBsAg). Jednotlivé formy HBsAg sdílejí C-konec a liší se N-koncovou částí. Nejkratší forma HBsAg nese označení S (small). M (medium) forma HBsAg obsahuje oproti S N-koncovou sekvenci preS2 o velikosti 55 AK. Nejdelší forma HBsAg nese označení L (large) a obsahuje na N-konci oproti M navíc sekvenci označenou jako preS1 o velikosti 108-119 AK (Lamontagne et al., 2016). preS1 se podílí na vazbě receptoru, kterým je lidský sodíko/taurocholátový kotransportní polypeptid (hNTCP), který je exprimován na povrchu lidských hepatocytů (Yan et al., 2012). Schopnost vazby receptoru HBsAg vykazuje tedy pouze nejdelší forma L. Po vazbě na receptor dochází k internalizaci virové částice HBV pomocí klatrinových váčků a transportu virového genomu do jádra (Cooper and Shaul, 2006; Huang et al., 2012). V jádře dochází k opravě relaxované kruhové DNA (rcDNA) za vzniku kovalentně uzavřené kruhové DNA (cccDNA). Na formaci cccDNA v jádře infikované buňky se podílí hostitelské enzymy, jako jsou polymeráza δ, endonukleáza flap 1 a DNA ligáza 1 (Kitamura et al., 2018; Wei and Ploss, 2020). cccDNA obsahuje hostitelské histony a perzistuje v infikovaných hepatocytech ve formě episomu (Bock et al., 2001). Jelikož cccDNA nemá replikační počátek a není se schopná replikovat v jádře s využitím hostitelských proteinů, slouží jako replikační templát nejdelší forma virové RNA označené jako pregenomová RNA (pgRNA), která je transkribována DNA dependentní RNA polymerázou II (Rall et al., 1983). pgRNA slouží jako templát pro virovou reverzní transkripci a také pro translaci kapsidového a P proteinu. Další virové RNA slouží jako templát pro translaci HBsAg, antigenu e (HBeAg) a proteinu X (Lamontagne et al., 2016). Vyjma virových částic je HBsAg sekretován z infikovaných buněk také ve formě tzv. subvirových partikulí (SVP). SVP mají dvě formy: 25 nm sférickou formu a 22 nm filamentózní formu. SVP jsou sekretovány v mnohem vyšším množství než infekční virové částice a jejich koncentrace v séru infikovaných osob může

přesahovat koncentraci virových částic i 10 000 krát (Glebe and Urban, 2007). Význam SVP patrně spočívá v úniku před imunitním systémem hostitele (Rydell et al., 2017). Dalším sekretovaným proteinem je HBeAg, který se podobně jako HBsAg podílí na modulaci imunitní odpovědi (Tian et al., 2016). Po translaci potřebného množství kapsidového proteinu a proteinů nutných k reverzní transkripci je pgRNA obalena společně s P proteinem a vzniká nukleokapsida (Lamontagne et al., 2016). V nukleokapsidě následně probíhá reverzní transkripce pgRNA. Významnou úlohu v reverzní transkripci hraje P protein obsahující několik funkčních domén. N-koncová doména P proteinu obsahuje tzv. terminální protein hrající roli v iniciaci reverzní transkripce. P protein dále obsahuje reverzně transkripční/polymerázovou doménu a doménu s aktivitou Rnázy H (Nassal, 2008). Finálním produktem reverzní transkripce pgRNA je rcDNA, která je v ER obalena lipidickou dvojvrstvou obsahující HBsAg a pomocí endosomálního sortovacího transportního komplexu (ESCRT) dochází k vypučení virových partikulí (Watanabe et al., 2007).



Obrázek 7: Schéma životního cyklu HBV. Po vazbě virové partikule na hNTCP dochází k internalizaci virové částice pomocí klatrinových váčků. Po uvolnění virové částice z endosomů dochází k částečnému rozvolnění virové nukleokapsidy a transportu do jádra. V jádře dochází k formování cccDNA z rcDNA pomocí hostitelských enzymů. cccDNA slouží v jádře jako templát pro virovou transkripci. Nejdelší transkript pgRNA slouží jako templát pro virovou reverzní transkripci a je společne s P proteinem zabalena do nově vznikající nukleokapsidy. Následuje krok reverzní transkripce, obalení nukleokapsidy v ER a vypučení nové virové partikule za pomoci ESCRT. Převzato a upraveno podle (Tong and Revill, 2016).

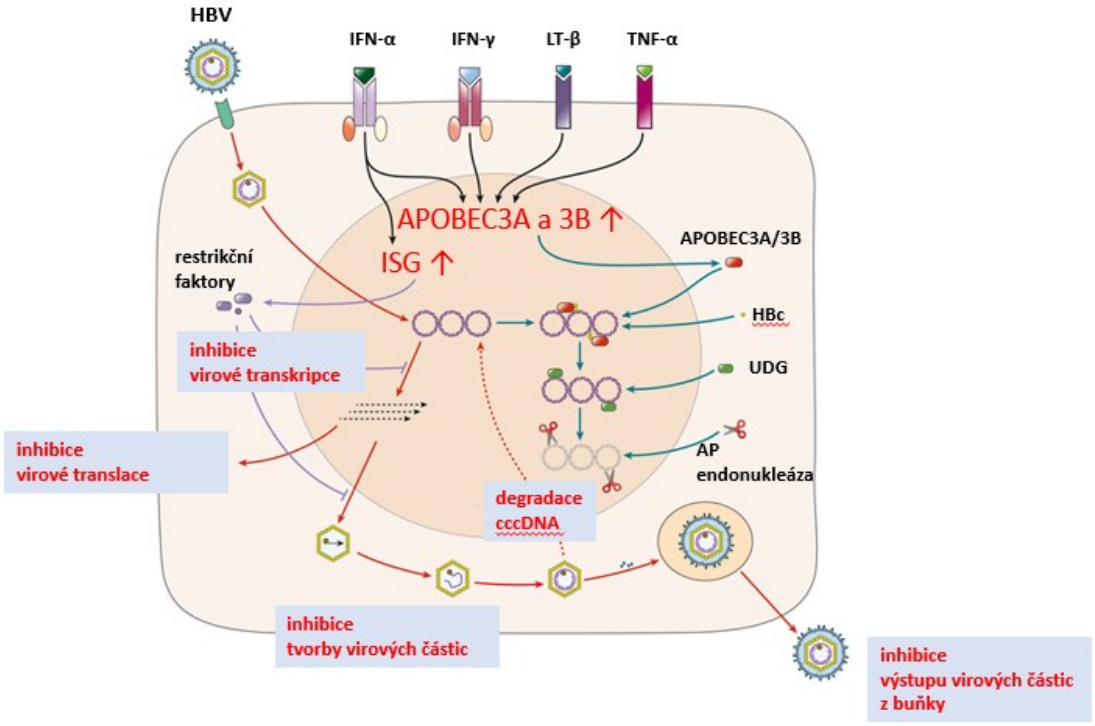
Pro zvládnutí akutní infekce HBV je potřeba aktivace adaptivní imunity, zejména polyspecifická indukce cytotoxických lymfocytů T (Thimme et al., 2003). Jelikož je HBV velmi slabým induktorem vrozené imunity, byl pro tento virus vytvořen koncept tzv. neviditelného viru, který je schopen uniknout před rozeznáním PRR (Wieland and Chisari, 2005). Mnoho studií prokázalo, že HBV infekce není rozeznána v primárních lidských hepatocytech (PHH), přestože jsou PHH vybaveny mnoha PRR a jsou schopné rozeznat jiné virové infekce (Mutz et al., 2018; Suslov et al., 2018). V játrech se kromě PHH vyskytuje velké množství imunitních buněk jako jsou kupferovy buňky, NK buňky a mnoho dalších, které se podílejí na rozeznání infekce HBV (Knolle and Thimme, 2014). Několik studií popsalo schopnost HBV modulovat funkci imunitních buněk v játrech (Faure-Dupuy et al., 2019; Tian et al., 2016; Wu et al., 2009). Pro příklad imunitní modulace lze uvést nedávno publikovanou práci od Faure-Dupuyové et al., ve které autoři popsali schopnost HBV inhibovat produkci IL-1β u jaterních makrofágů a měnit jejich prozánětlivý M1 fenotyp na proti-zánětlivý M2 fenotyp (Faure-Dupuy et al., 2019). Na ustavení chronické infekce se tak pravděpodobně podílí schopnost HBV unikat před receptory vrozené imunity i aktivní inhibice jaterních imunitních buněk.

2.5.1 Inhibiční účinek cytokinů na replikaci HBV a možné využití syntetických agonistů TLR pro léčbu chronické infekce HBV

Pro léčbu chronické infekce HBV je v současnosti schváleno použití nukleosidových nebo nukleotidových analogů (NUC) a rekombinantního IFN-α2a (Terrault et al., 2018). Základním předpokladem účinné léčby je degradace cccDNA v PHH. cccDNA vykazuje vysoký poločas života a přetrvává v PHH v nízkém počtu kopií (zhruba 10 kopií/hepatocyt) (Ko et al., 2018). cccDNA váže hostitelské histony a další nehistonové proteiny a perzistuje v jádře ve formě episomu (Bock et al., 2001). Vysoká stabilita a nízký počet kopií cccDNA představuje významný problém cílené léčby. NUC sice snižují produkci nových virových částic, ale příliš neovlivňují množství cccDNA v PHH. Také léčba rekombinantním IFN-α2a nevykazuje vysokou úspěšnost léčby a je spojena s vedlejšími efekty (Terrault et al., 2018).

Mnoho studií prokázalo inhibiční účinek IFN-I/III na virovou replikaci HBV v PHH (Lucifora et al., 2014; Robek et al., 2005; Xu et al., 2019), což potvrdilo i význam rekombinantního IFN-α2a pro léčbu chronické infekce HBV (Terrault et al., 2018). IFN-

I/III indukují v PHH zvýšení exprese ISG, které následně inhibují různá stadia životního cyklu HBV v PHH. Příkladem několika ISG schopných inhibovat HBV jsou například protein kináza R (PKR) inhibující virovou translaci (Park et al., 2011), ISG20 inhibující virovou replikaci (Liu et al., 2017), MxA inhibující tvorbu nukelokapsidy (Li et al., 2012) nebo BST2 inhibující vypučení virových částic HBV (Lv et al., 2015). IFN-I ovlivňují i acetylaci histonů vázaných na cccDNA (Belloni et al., 2012). Studie Lucifory et al. prokázala také schopnost IFN-α degradovat cccDNA v PHH. IFN-α indukuje v PHH zvýšení exprese enzymu APOBEC3A (apolipoprotein B mRNA editing enzyme catalytic subunit 3A). APOBEC3A deaminuje cytosiny přítomné v cccDNA, což vede ke vzniku apurinních/apyrimidinních míst v cccDNA a následně k její degradaci. IFN-α je tedy schopen indukovat necytotoxickou degradaci cccDNA v PHH (Lucifora et al., 2014). Působením IFN-α dochází v hepatocytech infikovaných HBV k epigenetickému potlačení virové transkripce a k redukci stability jeho genomu. Také IFN-III vykazují schopnost necytotoxické degradace cccDNA v PHH (Bockmann et al., 2019). Některé další studie ovšem vliv IFN-I na degradaci cccDNA nepotvrdily (Mutz et al., 2018; Niu et al., 2018; Shen et al., 2018). Necytolytická degradace cccDNA a inhibiční vliv na životní cyklus HBV byl popsán také pro další cytokiny jako jsou TNF-α a IFN-γ (Xia et al., 2016). Významnou limitací studie funkce jednotlivých cytokinů na životní cyklus HBV je nutnost užití vhodné buněčné linie. Mnoho studií prokázalo, že běžné hepatomové buněčné linie odvozené z nádorů jater jako HepG2, Huh7 a další, vykazují oproti PHH rozdílnou odpověď na cytokinovou stimulaci (Marozin et al., 2008; Shen et al., 2018). Nejvhodnější model pro studium cytokinové signalizace a vrozené imunitní odpovědi tak představují PHH.



Obrázek 7: Vliv IFN-I a dalších cytokinů na životní cyklus HBV v hepatocytech. IFN-α, IFN-γ, lymfotoxin beta (LT-β) a TNF-α zvyšují expresi APOBEC3A/3B v PHH. APOBEC3A/3B se váže na cccDNA a způsobuje deaminaci cytosinu, která je rozeznána uracyl-DNA glykozylázou (UDG). Apurinní/apyrimidinní (AP) endonukleáza rozeznává apurinní/apyrimidinní místa v cccDNA a přispívá tak k její degradaci. Kromě APOBEC3A indukuje IFN-α expresi ISG, které cílí virovou transkripci, translaci, tvorbu virových částic i samotné vypučení virových částic HBV. Převzato a upraveno podle (Block and Guo, 2016).

S ohledem na inhibiční vliv cytokinů na životní cyklus HBV byla testována schopnost několika syntetických agonistů TLR inhibovat replikaci HBV v hepatocytech. Syntetický agonista TLR7 GS-9620 prokázal vysokou účinnost inhibice replikace HBV na zvířecích modelech (Lanford et al., 2013; Menne et al., 2015). GS-9620 byl testován také na lidech. Podání GS-9620 chronickým HBV nosičům bylo dobře tolerováno, nicméně léčba chronické infekce HBV pomocí GS-9620 nevykazovala u lidí dostatečnou účinnost (Agarwal et al., 2018). Studie na PHH následně prokázala, že bezbuněčný supernatant z PBMC stimulovaných pomocí GS-9620 inhibuje replikaci HBV v PHH. Efekt GS-9620 na degradaci cccDNA v PHH nebyl ovšem prokázán (Niu et al., 2018). Také kombinace CpG ODN a nukleosidového analogu entecaviru na zvířecím modelu poukázala na možnost využití agonistů TLR v léčbě chronické infekce HBV (Meng et al., 2016). U některých

syntetických agonistů TLR byl prokázán také přímý inhibiční vliv na replikaci HBV v PHH, neboť PHH exprimují některé TLR. Syntetičtí agonisté TLR1/2 a TLR3 vykazovali inhibiční působení v PHH infikovaných HBV, které bylo částečně nezávislé na sekreci cytokinů stimulovanými PHH. Studie dále prokázala, že testovaní agonisté indukovali produkci cytokinů i u PBMC. Výsledný efekt inhibice tak patrně lze docílit jak přímou aktivací TLR v PHH, tak i nepřímou aktivací TLR v imunitních buňkách (Lucifora et al., 2018). Tento poznatek otevřel nové možnosti pro testování speciálně upravených duálních agonistů TLR, které vykazují širokou schopnost aktivace velkého množství imunitních buněk a mohou přímo aktivovat také PHH.

2.5.2 Vztah HBV a pDC

Význam jaterních pDC byl dlouho dobu předmětem diskuzí, a to i vzhledem k nízké frekvenci pDC v jaterní tkáni a s tím spojené obtížné izolaci a charakterizaci pDC. Jaterní prostředí je považováno za imunotolerantní a jaterní imunitní odpověď je velmi specifická, neboť játra jsou vystavena bakteriálním produktům jako je lipopolysacharid (Kubes and Jenne, 2018). Ve studii od Doyle et al. využili pro studium jaterních pDC metodu hmotností cytometrie a zjistili, že jaterní pDC jsou schopné produkovat vysoké množství IFN-I po stimulaci syntetickým agonistou TLR7/8. V porovnání s krevními pDC byla produkce IFN-I u jaterních pDC výrazně vyšší (Doyle et al., 2019). Na myším modelu byla popsána také imunotolerantní funkce pDC. Imunotolerantní funkci jaterních pDC potvrzují i data poukazující na asociaci poklesu frekvence jaterních pDC v případě odhojení jaterního transplantátu (Koda et al., 2019).

pDC by jako hlavní producenti IFN-I/III měly představovat důležitou složku antivirové obrany v případě HBV. Role pDC v rozeznání infekce HBV je ovšem značně nejasná a analýza pDC z chronických nosičů HBV přinesla protichůdné výsledky. Několik studií prokázalo, že frekvence pDC i jejich produkce cytokinů po izolaci a následné stimulaci *in vitro* u pacientů s chronickou infekcí HBV nevykazuje rozdíly v porovnání se zdravými dárci (Li et al., 2018; Tavakoli et al., 2008). Jiné studie naopak poukázaly na skutečnost, že pDC z chronických nosičů HBV produkují v porovnání se zdravými kontrolami nižší množství IFN-I a vykazují nižší expresi kostimulačních molekul po stimulaci *in vitro* (van der Molen et al., 2004; Xu et al., 2012). Většina studií je ovšem limitována nízkým počtem pacientů s chronickou infekcí HBV a analýzou pDC izolovaných z periferní krve. Ve studii od Martinetové et al. provedli autoři srovnání krevních i jaterních pDC získaných z pacientů s chronickou infekcí HBV. Frekvence

krevních ani jaterních pDC u chronických nosičů HBV se nelišila od frekvence pDC u zdravých kontrol. Zajímavé zjištění přinesla fenotypická analýza pDC, neboť jaterní i krevní pDC vykazovaly vyšší expresi kostimulačních molekul CD80 a CD40 oproti zdravým kontrolám. pDC z chronických nosičů HBV produkovaly po stimulaci pomocí CpG-A nižší množství IFN-I a vykazovaly nižší expresi kostimulační molekuly OX40L (ligand receptoru OX40) (Martinet et al., 2012). Inhibice produkce IFN-I u jaterních pDC byla potvrzena i v případě myšího modelu HBV (Hasebe et al., 2005).

Inhibiční vliv HBV na funkci pDC podporuje fakt, že v současnosti není k dispozici studie poukazující na schopnost pDC rozeznat virové částice HBV nebo buňky infikované HBV. Mnoho studií naopak potvrdilo schopnost HBV inhibovat signální dráhu TLR9 u pDC (Aillot et al., 2018; Woltman et al., 2011; Xu et al., 2012, 2009). V případě inhibice TLR9 u pDC bylo popsáno několik různých mechanismů inhibice. Jedna ze studií popsala schopnost HBsAg inhibovat TLR9 pomocí zvýšení hladiny supresoru cytokinové signalizace 1 (SOCS-1) u pDC. Autoři studie popsali také možnou vazbu HBsAg na BDCA-2. Aktivace signální dráhy BDCA-2 nebyla však plně prokázána (Xu et al., 2009). Studie od Woltmanové et al. prokázala vazbu HBsAg na BDCA-2 pouze v případě, že pDC byly předem aktivovány pomocí CpG-A. Studie nicméně potvrdila schopnost rekombinantního HBsAg a také HBeAg inhibovat TLR9 u pDC (Woltman et al., 2011). Dále byla popsána schopnost virových partikulí HBV vázat syntetické ligandy TLR9 a tím aktivně blokovat TLR9. Nicméně v práci byl popsán také inhibiční vliv rekombinantního HBsAg na pDC, který neměl schopnost vázat syntetické ligandy TLR9 (Aillot et al., 2018). Popsána byla i schopnost HBV snižovat expresi TLR9 u pDC (Xu et al., 2012). Na inhibici TLR9 u pDC by se mohla podílet také přítomnost inhibičních CpG sekvencí v genomu HBV, které mají schopnost se vázat na TLR9, ale neaktivují signální dráhu vedoucí k produkci IFN-I (Vincent et al., 2011). Přesný mechanismu inhibice pDC včetně zapojení RR v případě HBV vyžaduje další studie.

3. Cíle práce

Tématem disertační práce je studium vztahu pDC a vybraných obalených virů. pDC představují významné producenty IFN-I, kteří inhibují virovou replikaci indukcí ISG. Během vzájemné evoluce virů a jejich hostitelů došlo k selekci mnoha virů, které aktivně inhibují produkci IFN-I v pDC anebo unikají před rozeznáním vlastního genomu a jeho produktů prostřednictvím pDC. Základní cíle práce vychází z obousměrného působení pDC a vybraných obalených virů.

1) Objasnit vliv RR na pDC a možnosti farmakologického cílení signalizace RR.

- Srovnat z dostupné literatury vliv signalizace asociované s ITAM na signalizaci TLR u pDC, mDC a makrofágů.

- Objasnit roli signální dráhy MEK1/2-ERK v případě signalizace RR u pDC.

- Objasnit vliv HIV, jakožto možného aktivátoru (prostřednictvím TLR7) i inhibitoru (prostřednictvím gp120-BDCA-2), na fenotyp pDC u pacientů s HIV.

2) Objasnit vliv aktivovaných pDC na replikaci HBV v hepatocytech.

- Analýza vlivu pDC aktivovaných pomocí různých syntetitických ligandů TLR7/9 na replikaci HBV v PHH.

- Vliv cytokinového prostředí na množství cccDNA HBV v PHH.

4. Materiály a metody

Technické řešení jednotlivých projektů bylo prováděno na pracovišti virologické skupiny katedry Genetiky a Mikrobiologie ve výzkumném centru BIOCEV Přírodovědecké fakulty UK a ve virologické laboratoři s úrovní zabezpečení 3 (BSL3) v Ústavu organické chemie a biochemie AV ČR.

Výčet hlavních metod použitých pro sepsání disertační práce:

Izolace a kultivace imunitních buněk z periferní lidské krve

Imunofenotypická analýza buněk pomocí průtokové cytometrie

Analýza buněčných signálních drah pomocí průtokové cytometrie (PhosphoFlow) a pomocí Western blotu

Analýza buněčného cyklu pomocí průtokové cytometrie

Izolace a práce s HBV v laboratoři BSL3

Izolace a kultivace primárních lidských hepatocytů

Analýza produkce virových proteinů a produkce vybraných cytokinů metodou ELISA (enzyme-linked immuno sorbent assay)

Detekce virových nukleových kyselin metodikou qPCR

5. Výsledky

5.1 Publikované články vztahující se tematicky k disertační práci

Cross Talk between Inhibitory Immunoreceptor Tyrosine-Based Activation Motif-Signaling and Toll-Like Receptor Pathways in Macrophages and Dendritic Cells

Ivan Hirsch, Vaclav Janovec, Ruzena Stranska, Nathalie Bendriss-Vermare

Review Front Immunol. 2017 Apr 7;8:394. doi: 10.3389/fimmu.2017.00394. eCollection 2017.

IF_{2020} = 5,085

V tomto přehledovém článku (Příloha č. 1) jsme srovnali dopad receptorů asociovaných s ITAM na signalizaci TLR v pDC, mDCc a makrofázích. Z dostupné literatury a na základě předchozích výsledků naší pracovní skupiny vyplynulo, že aktivace receptorů asociovaných s ITAM u pDC vede k inhibici produkce IFN-I, zatímco v mDCc a makrofázích má tato signalizace za určitých podmínek pozitivní vliv na TLR. V přehledovém článku je dále popsána role RR u pDC v případě navození tzv. imunotolerantního stavu pDC a možnosti farmakologického cílení této signalizační dráhy.

Příspěvek autora 20 %. V tomto přehledovém článku jsem přispěl zejména korekcí textu a úpravě části přehledového článku a obrázků zabývající se signalizací TLR u pDC.

The MEK1/2-ERK Pathway Inhibits Type I IFN Production in Plasmacytoid Dendritic Cells

Vaclav Janovec, Besma Aouar, Albert Font-Haro, Tomas Hofman, Katerina Trejbalova, Jan Weber, Laurence Chaperot, Joel Plumas, Daniel Olive, Patrice Dubreuil, Jacques A Nunès, Ruzena Stranska, Ivan Hirsch

Front Immunol. 2018 Feb 26;9:364. doi: 10.3389/fimmu.2018.00364. eCollection 2018.

IF_{2020} = 5,085

Příspěvek autora 50%. V tomto článku (Příloha č. 2) jsme analyzovali vliv aktivace receptorů BDCA-2 a ILT-7 na produkci IFN-I u pDC. V rámci této práce jsem analyzoval zejména hladinu proteinu c-FOS po aktivaci receptoru BDCA-2 v pDC buněčné linii GEN2.2 a v primárních pDC. Navrhl jsem hypotézu, že za potenciací produkce IFN-I po

inhibici MEK1/2-ERK stojí zvýšená aktivita této dráhy u GEN2.2 z důvodu zapojení MEK1/2-ERK v proliferaci GEN2.2. Tuto hypotézu jsem následně potvrdil analýzou buněčného cyklu pomocí průtokové cytometrie u GEN2.2. Dále jsem analyzoval bazální množství proteinu c-FOS u primárních pDC a u buněk GEN2.2. V rámci této práce jsem provedl i analýzu množství c-FOS u pDC metodikou PhosfoFlow. Aktivně jsem se podílel na formování hlavního obsahu manuskriptu a významně jsem přispěl k úpravě obsahu a textu.

Expression of TIM-3 on Plasmacytoid Dendritic Cells as a Predictive Biomarker of Decline in HIV-1 RNA Level during ART

Albert Font-Haro, Vaclav Janovec, Tomas Hofman, Ladislav Machala, David Jilich, Zora Melkova, Jan Weber, Katerina Trejbalova, Ivan Hirsch

Viruses. 2018 Mar 28;10(4):154. doi: 10.3390/v10040154.

IF_{2020} = 3,816

Příspěvek autora 40 %. V tomto článku (Příloha č. 3) jsme analyzovali vliv antiretrovirové terapie (ART) na fenotyp pDC u pacientů s chronickou infekcí HIV. Bylo zjištěno, že fenotyp pDC není po ART plně obnoven. V rámci této publikace jsem se podílel na designu cytometrického panelu a analýze cytometrických dat. Dále jsem přispěl k úpravě textu dané publikace.

Toll-like receptor dual-acting agonists are potent inducers of PBMC-produced cytokines that inhibit hepatitis B virus production in primary human hepatocytes

Vaclav Janovec, Jan Hodek, Kamila Clarova, Tomas Hofman, Pavel Dostalik, Jiri Fronek, Jaroslav Chlupac, Laurence Chaperot, Sarah Durand, Thomas F Baumert, Iva Pichova, Barbora Lubyova, Ivan Hirsch, Jan Weber

Sci Rep. 2020 Jul 29;10(1):12767. doi: 10.1038/s41598-020-69614-7.

IF_{2020} = 3,998

Příspěvek autora 70%. V tomto článku (Příloha č. 4) jsme analyzovali vliv různých agonistů TLR na inhibici replikace HBV v hepatocytech. V rámci této publikace jsem se podílel na

izolaci PBMC z periferní krve. Izolaci a kultivaci primárních lidských hepatocytů z resekovaných lidských jater. Provedl jsem infekci PHH virem HBV. Dále jsem prováděl qPCR analýzu množství cccDNA, celkové DNA HBV, a stanovení množství virových proteinů HBe a HBs pomocí metody ELISA. Dále jsem v této práci navrhl metodiku srovnání efektu duálních agonistů TLR v případě stimulace PBMC. Aktivně jsem se podílel na obsahové formě manuskriptu i na úpravě textu.

5.2 Ostatní publikované články

VP1, the major capsid protein of the mouse polyomavirus, binds microtubules, promotes their acetylation and blocks the host cell cycle

Lenka Horníková, Martin Fraiberk, Petr Man, Václav Janovec, Jitka Forstová

FEBS J. 2017 Jan;284(2):301-323. doi: 10.1111/febs.13977. Epub 2017 Jan 9.

IF_{2020} = 4,392

Hepatitis B Core Protein Is Post-Translationally Modified through K29-Linked Ubiquitination

Hana Langerová, Barbora Lubyová, Aleš Zábranský, Martin Hubálek, Kristýna Glendová, Ludovic Aillot, Jan Hodek, Dmytro Strunin, Václav Janovec, Ivan Hirsch, Jan Weber

Cells. 2020 Nov 26;9(12):2547. doi: 10.3390/cells9122547.

IF_{2020} = 4,366

TLR4-Mediated Recognition of Mouse Polyomavirus Promotes Cancer-Associated Fibroblast-Like Phenotype and Cell Invasiveness

Vaclav Janovec, Boris Ryabchenko, Aneta Škarková, Karolína Pokorná, Daniel Rösel, Jan Brábek, Jan Weber, Jitka Forstová, Ivan Hirsch, Sandra Huérfano

Cancers (Basel). 2021 Apr 25;13(9):2076. doi: 10.3390/cancers13092076.

IF_{2020} = 6,126

6. Diskuse

pDC představují významné producenty IFN-I v lidském organismu a plní proto důležitou antivirovou úlohu v rámci imunitního systému. Produkce IFN-I musí být striktně regulována z důvodu možných patologií spjatých s nadměrnou produkcí IFN-I. Jedním z regulačních mechanismů produkce IFN-I u pDC jsou i RR přítomné na povrchu pDC. Mnoho virů využívá aktivaci RR jako strategii imunitního úniku. Tato disertační práce shrnuje nové poznatky získané studiem interakcí vybraných obalených virů s pDC. V mé práci jsem se věnoval hlavně interakci pDC s HBV a HIV-1, viry zodpovědnými za významná lidská chronická onemocnění, hepatitidu B a syndrom získané imunodeficience (AIDS).

Pro objasnění signalizace RR u pDC jsme provedli srovnání mechanismů vlivu regulačních receptorů asociovaných s ITAM na signalizaci TLR u pDC, mDC a makrofágů. U makrofágů byl v případě receptorů asociovaných s ITAM popsán tzv. switch model, kdy nízko-avidní aktivace receptorů asociovaných s ITAM vede k inhibici signalizace TLR a naopak vysoko-avidní aktivace receptorů asociovaných s ITAM vede k pozitivnímu efektu na signalizaci TLR. Centrálním regulátorem je přitom aktivita kinázy Syk, přičemž nízko-avidní aktivace receptorů vede u makrofágů k nízké aktivaci kinázy Syk oproti vysoko-avidní aktivaci receptorů, kdy dochází k plné aktivaci Syk (Ivashkiv, 2008). U pDC nebyl tento mechanismus pozorován, neboť nízko-avidní aktivace receptorů asociovaných s ITAM nevede k žádnému vlivu na TLR signalizaci (Jähn et al., 2010). Jak ukázala předchozí práce naší pracovní skupiny, vysoko-avidní aktivace RR u pDC vede k aktivaci Syk, ale na rozdíl od makrofágů dochází u pDC k inhibici signalizace TLR7/9 (Aouar et al., 2016). U mDC dochází po aktivaci receptorů asociovaných s ITAM k aktivaci Syk a následné aktivaci transkripčního faktoru NF-κB skrze CARD9-BCL9-MALT1 komplex (Kingeter and Lin, 2012). Vysoko-avidní aktivace receptorů asociovaných s ITAM tak u mDC podobně jako u makrofágů vede k pozitivnímu vlivu na signalizaci TLR. Při popisu signalizace BDCA-2 u pDC byla navržena možnost aktivace NF-κB (Cao et al., 2007). pDC exprimují i klíčový adaptorový protein CARMA1 (CARD-membrane associated guanylate kinase protein 1), který se podílí na aktivaci NF-κB (Roche et al., 2013). Nicméně, při aktivací receptorů asociovaných s ITAM nedochází u pDC k aktivaci NF-κB dráhy (Dental et al., 2012; Florentin et al., 2012). Z naší literární rešerše tedy vyplynulo, že RR asociované s adaptorovými molekulami obsahující ITAM negativně ovlivňují produkci IFN-I v lidských pDC a mechanismus působení receptorů asociovaných s ITAM na TLR

se liší v závislosti na buněčném typu. U myších pDC vede aktivace receptoru PDC-TREM (trigerring receptor expressed on myeloid cells-1), který asociuje s adaptorovou molekulou DAP12 obsahující ITAM, k posílení produkce IFN-I (Watarai et al., 2008). Nelze proto vyloučit, že jiné doposud nepopsané plazmatické receptory asociované s ITAM mohou pozitivně ovlivnit produkci IFN-I u pDC.

Jelikož viry využívají aktivaci RR jako mechanismus imunitního úniku, rozhodli jsme se testovat možnost farmakologického cílení signalizace RR s cílem obnovení produkce IFN-I v pDC. Naše výsledky prokázaly, že signální dráha MEK-ERK není esenciální pro tvorbu IFN-I u pDC. Inhibice MEK-ERK naopak vedla k inhibici produkce TNF-α u pDC. Vliv MEK-ERK na produkci cytokinů u pDC byl dále podpořen aktivací MEK-ERK pomocí forbol myristyl acetátu (PMA). PMA silně inhibovalo produkci IFN-I v pDC stimulovaných pomocí CpG-A, přičemž tento inhibiční efekt byl plně závislý na MEK-ERK. Samotné PMA bylo navíc schopné indukovat produkci TNF-α u pDC. Produkce TNF-α je pravděpodobně asociovaná se schopností PMA aktivovat nejen MEK-ERK, ale také NF-κB (Holden et al., 2008). Zajímavý fenomén potenciace produkce IFN-I byl pozorován v případě buněčné linie GEN2.2. Aktivace TLR9 v přítomnosti inhibitoru MEK1/2 vedla k významně vyšší produkci IFN-I než u kontroly, kde nebyl inhibitor přítomen. Tento potenciační efekt inhibitoru MEK1/2 ovšem nebyl pozorován u primárních pDC. Podrobnou analýzou bylo dále zjištěno, že MEK-ERK se podílí na proliferaci buněčné linie GEN2.2. Proliferující GEN2.2 tak mají patrně mnohem vyšší bazální aktivaci MEK-ERK oproti primárním pDC. Naše výsledky naznačují, že se MEK-ERK podílí na balancování množství produkce IFN-I a TNF-α u pDC.

Inhibice MEK-ERK dále vedla ke snížení inhibičního efektu BDCA-2 na produkci IFN-I. Několik studií prokázalo fosforylaci ERK1/2 po aktivaci BDCA-2 (Cao et al., 2007; Röck et al., 2007). Nicméně, naše výsledky prokázaly, že MEK-ERK se podílí i na inhibici produkce IFN-I u pDC. Dále jsme zjistili, že MEK-ERK indukuje zvýšení exprese a fosforylaci transkripčního faktoru c-FOS. Naproti tomu, inhibice MEK1/2 po aktivaci BDCA-2 nevedla k úplné inhibici exprese c-FOS. Jedním z možných vysvětlení je, že na zvýšení exprese c-FOS po aktivaci BDCA-2 se kromě MEK-ERK podílí i další signální dráhy. Nelze vyloučit ani MEK1/2 nezávislou aktivaci ERK, která byla popsána u neutrofilů (Simard et al., 2015). Základní nezodpovězenou otázkou však zůstává, zdali se c-FOS podílí na inhibici produkce IFN-I v lidských pDC. c-FOS negativně reguluje tvorbu IFN-I u myších makrofágů a mDC (Kaiser et al., 2009). Mechanismus regulace produkce

cytokinů pomocí ERK1/2 byl popsán i u monocytů. Po aktivaci TLR7 u monocytů dochází k fosforylaci ERK1/2 a následnému zvýšení hladiny proteinu FOSL1 (FOS-like antigen), který negativně reguluje produkci IFN-I (de Marcken et al., 2019). FOSL1 protein patří stejně jako c-FOS do proteinové rodiny FOS (Karamouzis et al., 2007). Jedním z možných mechanismů funkce c-FOS a FOSL1 je kompetice s aktivačním transkripčním faktorem 2 (ATF2) a c-JUN, kteří vytvářejí dimer aktivátorového proteinu 1 (AP-1) podílejícího se na tvorbě IFN-I (Maniatis et al., 1998). Různé složení dimerů AP1 vede k různé preferenci promotorů (Bakiri et al., 2002). U hepatocytů infikovaných HCV dochází po fosforylaci c-FOS k indukci miRNA-21, která cílí MyD88 a IRAK1 (Chen et al., 2013). Naše analýza ovšem neprokázala zvýšenou expresi miRNA-21 po aktivaci BDCA-2 u pDC (nepublikované výsledky). Objasnění role c-FOS u pDC proto vyžaduje další studium.

Inhibiční efekt MEK-ERK může být i zcela nezávislý na proteinech z rodiny FOS. Konstitutivně aktivní MEK1/2 inhibuje kinázu p38, která se podílí na aktivaci NF-κB (Carter and Hunninghake, 2000). Kináza p38 je klíčová pro tvorbu IFN-I v pDC (Osawa et al., 2006), což prokázaly i naše výsledky získané použitím inhibitoru p38. MEK-ERK může navíc ovlivnit i samotnou signalizaci IFN-I. Aktivace IFNAR u pDC představuje pozitivní signalizační smyčku, neboť silně potencuje produkci IFN-I (Kim et al., 2014). Vliv signalizace RR na aktivitu p38 a IFNAR proto vyžaduje další studium. Inhibiční efekt MEK1/2 na interferonovou dráhu byl popsán i v jiných buněčných typech. Inhibice MEK1/2 u makrofágů vedla ke zvýšení množství IRF1, což mělo za následek zvýšení interferonové odpovědi (Yang and Ding, 2019). Zvýšená aktivace MEK-ERK byla popsána také u nádorových linií a byla asociována s nižší produkcí ISG (Christian et al., 2012). MEK-ERK se podílí i na indukci tolerance u T lymfocytů (Chen et al., 1999). Souhrnně lze tedy říct, že MEK-ERK se podílí na inhibici IFN-I a indukci ISG nejen u pDC, ale i v dalších buněčných liniích. Získané výsledky, spolu s existencí vysoce specifických a málo toxických inhibitorů MEK (PD0325901 je selektivní alosterický inhibitor MEK1/2 s IC_{50}=0.33 nM) poukazují na možnost cílení signální dráhy MEK-ERK za účelem obnovení funkce pDC.

Dále jsme zkoumali vliv chronické infekce HIV na fenotyp pDC. Zjistili jsme, že frekvence pDC v periferní krvi je u pacientů s HIV oproti zdravým donorům výrazně nižší. Nižší zastoupení pDC v periferní krvi u pacientů s HIV bylo prokázáno i v dalších studiích (Chehimi et al., 2007; Finke et al., 2004; Siegal et al., 2001). Na snížení množství pDC v periferní krvi u pacientů s HIV se pravděpodobně podílí 2 mechanismy, a to redistribuce

pDC do lymfatických tkání a přímý apoptotický vliv HIV na pDC (Boasso and Shearer, 2008). Naší základní otázkou bylo, jestli po aktivní retrovirové terapii (ART) u pacientů s HIV dojde k obnovení množství pDC a jestli snížení množství RNA HIV-1 po ART je asociováno se změnou fenotypu pDC. Analýza fenotypu pDC pomocí průtokové cytometrie prokázala, že exprese povrchového TIM-3 je zvýšena u pDC pacientů s HIV. TIM-3 vazbou IRF7 a regulační podjednotky p85 PI3K v lysozomech inhibuje produkci IFN-I u pDC a je silně produkován po aktivaci TLR7/9 u pDC. TIM-3 představuje marker tzv. vyčerpaných pDC, které vykazují nízkou produkci IFN-I u chronických HIV pacientů (Schwartz et al., 2017). Vyšší expresi jsme naměřili také v případě TRAIL, který je podobně jako TIM-3 produkován ve vyšší míře po aktivaci TLR7/9 u pDC (Chaperot et al., 2006; Hardy et al., 2007). Zvýšená exprese TIM-3 a TRAIL u pDC podporují hypotézu, že chronická infekce HIV vede k perzistentní aktivaci pDC (Barrat and Su, 2019). Při aktivaci TLR7/9 u pDC dochází ke snížení množství povrchového receptoru BDCA-2 (Wu et al., 2008). Lze tedy předpokládat, že pDC pacientů s chronickou infekcí HIV budou společně s vysokou expresí TIM-3 a TRAIL vykazovat nižší množství povrchového receptoru BDCA-2. Nicméně, naše výsledky naopak prokázaly zvýšení povrchového receptoru BDCA-2 u pDC pacientů s chronickou infekcí HIV. Studie Kaushika et al. ukázala, že množství BDCA-2 na povrchu pDC je u pacientů s infekcí HIV stejná jako u zdravých jedinců. Po stimulaci TLR7 ovšem došlo k výraznému snížení BDCA-2 u pDC získaných z pacientů infikovaných HIV (Kaushik et al., 2013). Naproti tomu jiná studie ukázala, že množství povrchového BDCA-2 je nižší u pDC lokalizovaných v lymfatických uzlinách u pacientů s HIV (Lehmann et al., 2010). Vysoká exprese BDCA-2 u tzv. vyčerpaných pDC by mohla být způsobena rozdílnou aktivací TLR7. Zatímco syntetické ligandy TLR7/9 a virus chřipky indukují velmi rychlou produkci IFN-I, HIV indukuje fosforylaci kinázy Syk s následnou opožděnou produkcí IFN-I (Lo et al., 2012). Není přitom jasné, jestli je do aktivace Syk kinázy u pDC v případě infekce HIV zapojen BDCA-2. Nicméně, Syk hraje roli v pozitivní i negativní regulaci TLR7/9 (Aouar et al., 2016). Role BDCA-2 a dalších RR v případě chronické infekce HIV tak vyžaduje další studium.

Dále jsme analyzovali efekt 9 měsíců trvající ART na fenotyp pDC. Naše data potvrdila výsledky několika dalších studií (Chehimi et al., 2007; Finke et al., 2004; Pacanowski et al., 2004), ukazující částečné obnovení množství krevních pDC po ART. Trend normalizace exprese po ART byl pozorován také v případě BDCA-2, TRAIL a TIM-3. Pokles RNA HIV v prvních třech měsících ART navíc negativně koreloval s mírou

exprese TIM-3 u pDC. Některé studie poukázaly na možnost využití množství pDC v periferní krvi jako prediktivního znaku účinnosti ART u pacientů s HIV, neboť vyšší množství pDC po ART korelovalo s nižší virovou náloží HIV (Pacanowski et al., 2004, 2001). Naše výsledky naznačují, že nejen množství pDC v periferní krvi, ale také množství TIM-3 u pDC lze využít jako prediktivní ukazatel účinnosti ART. Role TIM-3 jako prediktivního znaku bude vyžadovat další studium, neboť naše studie byla limitována množstvím pacientů infikovaných HIV. Dále není zřejmé, zda obnovení množství pDC a snížení TIM-3 vedlo k obnovení funkce produkce IFN-I, neboť v naší studii jsme neměřili produkci IFN-I po stimulaci TLR7/9 u pDC. Nicméně, pDC mohou po ART patrně hrát pozitivní i negativní roli v případě následné kontroly replikace HIV (Cohn and Deeks, 2020). Na myším modelu chronické infekce HIV bylo demonstrováno, že trvalá produkce IFN-I se podílí na vzniku imunopatologií (Su, 2019). Jednou z navržených a testovaných strategií boje proti chronické infekci HIV je proto i cílení produkce IFN-I (Barrat and Su, 2019). Naše výsledky poukázaly na zachování regulačního receptoru BDCA-2 u pDC získaných z pacientů s chronickou infekcí HIV, a tedy na možnost cílení toho receptoru.

Posledním řešeným tématem této disertační práce bylo objasnění vztahu pDC a HBV. Několik studií potvrdilo, že pDC nejsou schopné rozeznat HBV a nedochází tak produkci IFN-I (Aillot et al., 2018; Vincent et al., 2011; Woltman et al., 2011; Xu et al., 2009). Produkce IFN-I je přitom klíčová pro potlačení replikace HBV a rekombinantní IFN-α2a je využíván k léčbě chronické infekce HBV (Lamontagne et al., 2016). Naše výsledky potvrdily, že pDC po expozici volných virových partikulí HBV neprodukují IFN-I a k aktivaci pDC nedochází ani po kokultivaci pDC s hepatocyty infikovanými HBV. Nepublikovaná data z naší laboratoře poukazují na aktivní inhibici produkce IFN-I u pDC a schopnost HBV ovlivnit funkci pDC je nadále předmětem zájmu naší laboratoře. Vzhledem k důležitosti pDC, jakožto významných producentů IFN-I, jsme se rozhodli otestovat schopnost aktivovaných pDC inhibovat virovou replikaci HBV v hepatocytech.

Vybrali jsme několik syntetických agonistů TLR7/9 schopných aktivovat produkci IFN-I u pDC. Vyjma agonistů schopných aktivovat jeden TLR jsme testovali i tzv. duální agonisty schopné aktivovat zároveň dva TLR. Byla analyzována schopnost jednotlivých syntetických agonistů indukovat produkci cytokinů u PBMC a dále efekt získaných bezbuněčných supernatantů z aktivovaných PBMC na sekreci HBeAg a HBsAg u PHH infikovaných HBV. Základní statistickou analýzou jsme zjistili, že hlavním hybatelem inhibice sekrece HBeAg a HBsAg u PHH není množství IFN-I, ale spíše zastoupení

širokého spektra vybraných cytokinů. Nejvyšší úrovně inhibice sekrece HBeAg a HBsAg bylo dosaženo při použití syntetických duálních agonistů R848 (TLR7/8) a CL413 (TLR2/7), které indukovaly kromě produkce IFN-I také produkci IFN-γ a prozánětlivých cytokinů IL-6, TNF-α a IL-12. Široké spektrum cytokinů v případě R848 a CL413 je patrně způsobeno schopností těchto duálních agonistů aktivovat kromě pDC také monocyty a mDC (de Marcken et al., 2019; Hémont et al., 2013). Naproti tomu syntetický agonista CpG-A (TLR9) indukoval u PBMC vysokou produkci IFN-I a IFN-III, produkce prozánětlivých cytokinů byla ovšem nízká. Významný vliv IFN-I na inhibici sekrece HBeAg a HBsAg byl nicméně potvrzen použitím agonistů CL264 (TLR7) a CpG-B (TLR9), které indukovaly pouze produkci prozánětlivých cytokinů a vykazovaly nízkou efektivitu inhibice. Na výslednou inhibici sekrece HBeAg a HBsAg u PHH infikovaných HBV tak zřejmě má vliv kumulativní efekt různých cytokinů. Naše výsledky poukazují na skutečnost, že využití polyspecifických agonistů TLR cílících nejen na aktivaci pDC může představovat efektivnější alternativu v případě léčby chronické infekce HBV.

Aktivované pDC produkují vyjma IFN-I také IFN-III (Yin et al., 2012), a proto jsme dále analyzovali vliv jednotlivých typů interferonů na inhibici sekrece HBeAg a HBsAg u PHH infikovaných HBV. Za pomoci neutralizačních protilátek proti IFNAR a IFNLR jsme zjistili, že hlavní inhibiční roli zřejmě plní IFN-I a nedochází ke kumulativnímu efektu s IFN-III. IFN-I a IFN-III indukují expresi velmi podobných ISG, avšak s různorodou kinetikou. IFN-I zprostředkovávají velmi rychlou antivirovou odpověď s vysokou expresí ISG, zatímco IFN-III zajišťují pomalejší nástup exprese ISG s nižší mírou exprese (Pervolaraki et al., 2018). Lze tedy předpokládat, že v námi sledovaném experimentu jsme pozorovali rychlý antivirový vliv IFN-I. Nelze vyloučit, že v případě dlouhodobějšího působení IFN-I a IFN-III se uplatní také IFN-III, neboť IFN-III podléhá jiné negativní regulaci než IFN-I (Stanifer et al., 2019). Je nutné dodat, že pro objasnění různého vlivu IFN-I a IFN-III na inhibici HBV v hepatocytech by bylo nutné použít buněčné modely deficientní v IFNAR nebo IFNLR, neboť námi použitá neutralizační protilátka proti IFNAR nevykazovala 100% účinnost inhibice ani v případě použití vysokých koncentrací.

Základním předpokladem pro efektivní léčbu chronické infekce HBV zůstává eliminace cccDNA. V naší studii žádný z použitých syntetických agonistů TLR nevykazoval schopnost indukce cytokinového prostředí po stimulaci PBMC, které by vedlo ke snížení množství cccDNA v PHH infikovaných HBV. Pozorován byl úbytek celkové DNA HBV, což potvrzuje inhibiční efekt cytokinového prostředí na virovou replikaci HBV

v PHH. Přestože byl popsán mechanismus, kterým IFN-I pomocí zvýšení exprese APOBEC3A snižují množství cccDNA v PHH (Lucifora et al., 2014), některé studie efekt IFN-I na degradaci cccDNA nepotvrdily (Mutz et al., 2018; Niu et al., 2018; Shen et al., 2018). Schopnost degradace cccDNA byla publikována také při použití IFN-γ, TNF-α a IFN-III (Bockmann et al., 2019; Xia et al., 2016). Cytokiny využívající různé signální dráhy tak mají schopnost snižovat množství cccDNA. Velkým otazníkem ovšem zůstává, zdali společným působením těchto cytokinů dochází k zesílení degradace cccDNA, nebo zdali mohou některé cytokiny naopak degradaci cccDNA ovlivnit negativně. V naší práci jsme testovali zejména přirozené cytokinové prostředí vyprodukované po stimulaci vybraných TLR u PBMC. Je potřeba zmínit, že neznáme přesně složení jednotlivých bezbuněčných supernatantů získaných stimulací PBMC, neboť jsme měřili pouze několik vybraných cytokinů. Naše výsledky jsou v souladu se studií Niu et al., ve které použití bezbuněčného supernatantu získaného stimulací PBMC pomocí GS-9620 nevedlo ke snížení cccDNA (Niu et al., 2018). V jiné studii byl však inhibiční vliv bezbuněčného supernatantu získaného po stimulaci PBMC pomocí syntetických agonistů TLR na množství cccDNA v PHH prokázán (Lucifora et al., 2018). Dalším vysvětlením rozdílných výsledků může být rozdílný přístup kvantifikace cccDNA pomocí qPCR (Li et al., 2017) a patrně i standardizace kultivace PHH (Lucifora et al., 2018, 2014; Niu et al., 2018). Podobný výsledek se studií Niu et al. lze v našem případě vysvětlit použitím stejné metodiky pro kultivaci PHH i pro kvantifikaci cccDNA pomocí qPCR. Limitací naší studie je neprovedení kvantifikace cccDNA metodou Southern blot z důvodu nízkého množství materiálu. Kromě degradace cccDNA může IFN-I ovlivnit kompozici cccDNA také pomocí vlivu na modifikaci histonů (Belloni et al., 2012; Yuan et al., 2020). Jelikož došlo k významnému poklesu sekrece HBe, HBs a také celkové DNA HBV bez poklesu hladiny cccDNA, lze předpokládat, že cytokinové prostředí indukovalo histonové modifikace cccDNA.

7. Shrnutí

Tato disertační práce shrnuje originální poznatky získané studiem vztahu pDC a vybraných virů zodpovědných za rozvoj chronických onemocnění. Naše výsledky prokázaly, že signalizace MEK-ERK se podílí na inhibici produkce IFN-I v pDC. MEK-ERK se podílí také na inhibici produkce IFN-I u pDC i v případě aktivace RR. Nicméně, v případě RR nevedla inhibice MEK-ERK k plnému obnovení produkce IFN-I. MEK-ERK je tedy pouze jednou komponentou signální dráhy RR a objasnění inhibičního mechanismu této dráhy vyžaduje další studium. V porovnání s jinými buněčnými typy jako jsou makrofágy a mDC je efekt RR asociovaných s ITAM vysoce specifický pro pDC. Nicméně, vliv aktivace MEK-ERK na produkci IFN-I a ISG má obecný charakter, neboť byl popsán u mnoha různých buněčných typů. Objasnění mechanismu funkce MEK-ERK na produkci IFN-I tak do budoucna představuje důležitý krok s cílem možného farmakologického cílení této signální dráhy nejen u pDC.

Analýzou fenotypu pDC získaných z pacientů s chronickou infekcí HIV jsme zjistili, že pDC vykazují vyšší expresi povrchových molekul TIM-3 a TRAIL. Oba tyto znaky poukazují na chronickou aktivaci pDC u pacientů s HIV a také na tzv. vyčerpaný fenotyp pDC, při kterém je snížena produkce IFN-I po stimulaci TLR7/9. Exprese BDCA-2 byla u pDC získaných z pacientů s HIV překvapivě vyšší než u zdravých kontrol. Jedním z možných vysvětlení zachování povrchové exprese BDCA-2 by mohl být vliv HIV na aktivaci kinázy Syk. Naše výsledky dále poukázaly na skutečnost, že ART vede k pouze částečnému obnovení množství i fenotypu pDC. U pacientů jejichž pDC vykazovaly vysokou expresi TIM3 byl navíc po ART pokles virové nálože HIV nižší. Úroveň vyčerpání pDC tak patrně lze u pacientů s HIV využít jako potenciální prediktivní ukazatel efektivity ART.

Posledním zkoumaným virem byl HBV. Jelikož tento virus velmi efektivně uniká před rozeznáním pDC, rozhodli jsme se otestovat efekt pDC aktivovaných pomocí různých syntetických ligandů TLR7/9 na virovou replikaci HBV v hepatocytech. Aktivované pDC efektivně inhibovaly replikaci HBV v hepatocytech, přičemž efekt inhibice byl zprostředkován zejména sekrecí IFN-I. Naše výsledky dále prokázaly, že využití syntetitických ligandů schopných aktivovat dva různé TLR vedlo po stimulaci PBMC k produkci cytokinového prostředí, které velmi efektivně inhibovalo replikaci HBV. Aktivace širšího repertoáru imunitních buněk tak patrně představuje mnohem efektivnější možnost inhibice chronické infekce HBV než v případě zacílení pouze na pDC. Nicméně,

pDC mohou kromě produkce IFN-I plnit také roli antigen prezentujících buněk. Vzhledem k nedostupnosti efektivní terapie proti chronické infekci HBV představuje objasnění vztahu pDC a HBV jedním z hlavních témat nejen naší laboratoře.

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9. Přílohy

9.1 Příloha č. 1

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REVIEW
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Cross Talk between Inhibitory Immunoreceptor Tyrosine-Based Activation Motif-Signaling and Toll-Like Receptor Pathways in Macrophages and Dendritic Cells

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The innate immune cells sense microbial infection and self-ligands by pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs) and regulatory receptors (RRs), associated with immunoreceptor tyrosine-based activation motif (ITAM). Rapid activation and concerted action of PRRs signaling and feedback inhibitory mechanisms must be engaged to ensure the host defense functions and to prevent cytotoxicity associated with excessive activation. ITAM-associated RRs can generate stimulatory or, paradoxically, inhibitory signals. The network of ITAM-associated RR, together with TLR-signaling pathways, are responsible for immunogenic or tolerogenic responses of macrophages and dendritic cells to their microenvironment. In macrophages, TLR4 signaling is inhibited by low-avidity ligation of ITAM-associated receptors, while high-avidity ligation of ITAM-associated receptors results in potentiation of TLR4 signaling together with resistance to extracellular cytokine microenvironment signals. In contrast to macrophages, TLR7/9 signaling in plasmacytoid DCs (pDCs) is inhibited by high-avidity ligation of ITAM-associated RR, while low-avidity ligation does not show any effect. Surprisingly, interference of ITAM-associated receptor signaling with TLR pathways has not been

Abbreviations: BCR, B-cell receptor; BDCA-2, blood dendritic cell antigen 2; BLNK, B-cell linker protein; BTK, Bruton's tyrosine kinase; CD2AP, CD2-associated protein (AP); cDC, conventional dendritic cell; DAG, diacylglycerol; DAP12, DNAX activation protein 12; DC, dendritic cell; DCIR, dendritic cell immunoreceptor; ECM, extracellular matrix; FcRs, Fc receptors; IC, immune complexes; IL-6, interleukin-6; IFN, interferon; ILT7, immunoglobulin-like transcript; IRAK1/4, interleukin-1 receptor-associated kinase 1/4; IRF7, interferon-regulatory factor 7; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; LLR, lectin-like receptors; MDL-1, myeloid DAP12-associated lectin-1; MICL, myeloid C-type lectin-like receptor; Mincle, macrophage-inducible C-type lectin; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor kappa B; pDC, plasmacytoid DC; PI3K, phosphatidylinositol 3-OH kinase; PLCγ2, phospholipase Cγ 2; PRRs, pathogen recognition receptors; RF, rheumatoid factor; RGD, arginine–glycyl–aspartic acid motif; RRs, regulatory receptors; SHIP, SH2-domain-containing inositol phosphatase-1; R848, Resiquimod; SHP1, SRC-homology-2 (SH2)-domain-containing protein tyrosine phosphatase 1; TAK1, transforming growth factor β-activated kinase 1; TCR, T-cell receptor; TLRs, toll-like receptors; TNF-α, tumor necrosis factor-α; Treg, regulatory T cells T cells; TRAF3/6, tumor necrosis factor receptor-associated factors 3 and 6; TREM2, triggering receptor expressed on myeloid cells 2; SYK, spleen tyrosine kinase.

reported in conventional dendritic cells. Here, we present an overview of molecular mechanisms acting at the crossroads of TLR and ITAM-signaling pathways and address the question of how the high-avidity engagement of the ITAM-associated receptors in pDCs inhibits TLR7/9 signaling. Cellular context and spatiotemporal engagement of ITAM- and TLR-signaling pathways are responsible for different outcomes of macrophage versus pDC activation. While the cross-regulation of cytokine and TLR signaling, together with antigen presentation, are the principal functions of ITAM-associated RR in macrophages, the major role of these receptors in pDCs seems to be related to inhibition of cytokine production and reestablishment of a tolerogenic state following pDC activation. Pharmacologic targeting of TLR and ITAM signaling could be an attractive new therapeutic approach for treatment of chronic infections, cancer, and autoimmune and inflammatory diseases related to pDCs.

Keywords: plasmacytoid dendritic cell, conventional dendritic cells, macrophage, toll-like receptors, regulatory receptors, immunoreceptor tyrosine-based activation motif-associated receptor, B cell receptor-like signaling

INTRODUCTION

Macrophages and dendritic cells (DCs) play a major role in initiating and sustaining innate and adaptive immune responses and are the nexus at which immune stimulation or suppression occurs (1–5). The innate immune cells sense microbial infection and self-ligands such as damaged or altered self, including dead cells, by pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs) and lectin-like receptors (LLRs), also called C-type lectin receptors (6). Rapid activation and concerted action of PRRs signaling is needed to ensure the host defense functions after infectious challenge or tissue damage. PRR agonists and secreted cytokines and chemokines are the drivers and the major regulators of fine-tuned innate immune responses. Concomitantly, feedback inhibitory mechanisms must be engaged to prevent cytotoxicity associated with excessive activation of the innate immune cells (7). Thus, TLRs that confer functional specificity to macrophages and DC subsets trigger intracellular signaling cascades that result in the secretion of interferons (IFNs) and pro-inflammatory cytokines and activation of host defense programs necessary for innate or adaptive immune responses. The same cells also specifically express immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors that can modulate TLR-signaling pathways (3, 8, 9). The conserved ITAM-signaling motif, with a consensus sequence YXXL/I–X_{6-8}–YXXL/I (where X denotes any amino acid), is present in the cytoplasmic tail of transmembrane adaptor molecules associated with multiple receptors. Initially discovered ITAM-associated receptors, including the T-cell receptor, B-cell receptor (BCR), and Fc receptors (FcRs), were shown to induce phosphorylation of the tyrosines within the ITAMs, to recruit Syk tyrosine kinases, and to activate the immune cell. More recent studies have shown that some ITAM-associated receptors mainly in the innate immune cells efficiently inhibit downstream signaling triggered by other types of PRRs.

Here, we present an overview of molecular mechanisms acting at the crossroads of TLRs and regulatory receptors (RRs) signaling and address the question of how the engagement of the ITAM-associated receptors in macrophages and two subtypes of DCs, conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs), inhibits cytokine and TLR7/9 signaling. We compare ITAM-mediated inhibitory mechanisms and function of the ITAM-associated receptors in these cell types. We focused our review on the neglected observation that TLR signaling in pDCs is inhibited by high-avidity engagement of the ITAM-associated RRs; while in macrophages, it is inhibited by low-avidity engagement of these receptors.

On the basis of this comparison, we assess the functions of the ITAM-associated receptors in those cells types. We hypothesize that while antigen presentation and cross-regulation of cytokine and TLR signaling are the principal functions of ITAM-associated receptors in macrophages, the major role of these receptors in pDCs is the inhibition of cytokine production and reestablishment of a tolerogenic state following pDC activation.

IMMUNOGENIC AND TOLEROGENIC RECEPTORS OF DCs

Plasmacytoid DCs are a highly specialized subset of DCs that function as sentinels for viral infection and cancer. They are responsible for production of type I and III IFNs, IFN-I (namely IFN-α, β, and ω) and IFN-III (IFN-λ1, λ2, λ3, and λ4 also called IL-29, IL-28A, IL-28B, and IL-28C), pro-inflammatory cytokines, and antigen presentation (**Figure 1A**). pDCs are able to detect genetic material of viruses with a subset of nucleotide-sensing TLRs localized in the endosomal compartment: TLR7, which recognizes single-stranded RNA, and TLR9, which recognizes DNA. TLR7 also recognizes synthetic imidazoquinoline components, for example Resiquimod (R848), whereas TLR9 recognizes synthetic CpG oligonucleotides. Ligation of TLR9 with aggregating CpG-A oligonucleotides in the early endosomes triggers the adaptor protein 3-dependent MyD88-IRF7 pathway that includes TLR adaptor MyD88, interleukin-1 receptor-associated kinase 1/4 (IRAK1/4), tumor necrosis factor receptor-associated factors 3 and 6 (TRAF3/6), and interferon-regulatory factor 7 (IRF7), and that results in the type I IFN production (3, 5, 10, 11) (**Figure 1A**). Activated IRF7, which is constitutively expressed in pDCs,

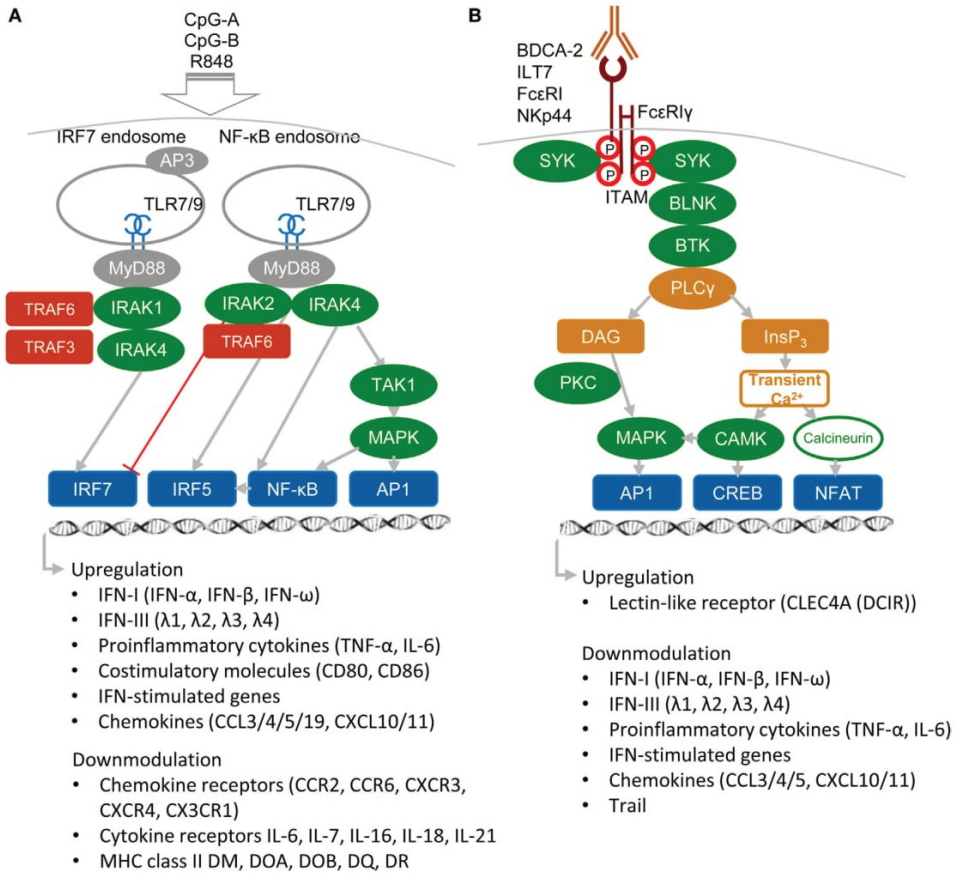

FIGURE 1 | Activatory signaling pathways control TLR7/9 signaling in plasmacytoid DCs (pDCs) (simplified view). **(A)** Ligation of TLR7 or TLR9 with their agonists, CpG-A or CpG-B, induces different types of signaling. Aggregating CpG-A is transported in the presence of adaptor protein 3 (AP3) to the interferon-regulatory factor 7 (IRF7) endosomes, where a signalosome composed of IRAK1, IRAK4, TRAF3, and TRAF6 is formed. IRF7 signalosome activates production of interferons (IFNs)-I (IFN-α/β) and IFN-III (IFN-λ). In contrast, transcriptome studies showed that CpG-A downmodulates expression of some chemokine and cytokine receptors and MHC class II molecules (16). Monomeric CpG-B is transferred to the NF-κB endosomes, where a signalosome is formed from the set including IRAK4, TRAF6, transforming growth factor β-activated kinase 1 (TAK1), and NF-κB/MAPK/IRF5, which leads to maturation of pDCs and formation of pro-inflammatory cytokines and chemokines. IRAK2 suppresses IFN-I/III production but positively controls production of pro-inflammatory cytokines (3, 5, 16). **(B)** Regulatory signaling pathways. Crosslinking of regulatory receptors, such as blood dendritic cell antigen 2 (BDCA-2), ILT-7, FcεRI, or NKp44, induces activation signals that lead to phosphorylation of tyrosines (shown by red circles) within the FcεRIγ immunoreceptor tyrosine-based activation motif (ITAM) and to recruitment of spleen tyrosine kinase (SYK) kinase. FcεRIγ-mediated B-cell receptor (BCR)-like signaling involving SYK, B-cell linker protein (BLNK), Bruton's tyrosine kinase (BTK), and PLCγ results in hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($InsP_3$). DAG activates protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), which contribute to activation of activator protein (AP1) and NF-κB. $InsP_3$ leads to transient release of intracellular Ca^{2+} stores followed by activation of calmodulin-dependent kinase (CAMK) and cyclic-AMP-responsive-element-binding protein. In parallel, calcineurin contributes to activation of the nuclear factor of activated T cells (NFAT). Transcriptome studies showed that BDCA-2 downmodulates expression of IFN-I, IFN-III, some chemokines and cytokines, and IFN-stimulated genes (16).

translocates to the nucleus and, together with ATF-2, c-Jun, and nuclear factor kappa B (NF-κB) subunits p50 and RelA, initiates the transcription of IFN-I (12). Furthermore, it has been demonstrated that TLR9-mediated induction of transforming growth factor β-activated kinase 1 (TAK1) and of inhibitor of nuclear factor κB kinase subunit β, followed by the IFN-β-stimulated activation of the JAK-STAT1/2 pathway, are essential for production of IFN-α (13). This second loop of IFN-I signaling induced by IFN-β secreted by pDCs triggers a robust IFN-I/III response and expression of IFN-stimulated genes, and it can be blocked by mAbs against secreted IFN-I or IFN-α/β receptor. In contrast to IRF7-mediated production of IFN-I, monomeric CpG-B oligonucleotides are transferred to an endolysosomal compartment where they activate the MyD88–NF-κB pathway that triggers expression of mitogen-activated protein kinases (MAPKs) and IRF5 (14, 15) (**Figure 1A**). Both, NF-κB and MAPKs, stimulate secretion of chemokines and of the pro-inflammatory cytokines interleukin-6 and tumor necrosis factor-α (TNF-α) and stimulate

expression of co-stimulatory molecules, such as CD80 (B7.1) and CD86 (B7.2).

In addition to nucleotide-sensing TLRs, pDCs also recognize pathogens through a battery of cell surface RRs, including FcRs and LLRs. The principal function of these RRs on pDCs is to facilitate antigen capture and presentation and to prevent aberrant immune responses by modulating production of IFN-I and pro-inflammatory cytokines (3, 5, 11) (**Figure 1B**). RRs deliver their signal through immunoreceptor tyrosine-based inhibition motif (ITIM) or through ITAM-associated adaptors, like the γ-chain of FcεRIγ or DNAX activation protein 12 (DAP12). Among ITAM-signaling receptors, blood dendritic cell antigen 2 (BDCA-2, CD303, CLEC4C) (17, 18), immunoglobulin-like transcript (ILT7, CD85g) (19, 20), and FcεRI (21) signal through FcεRIγ, while NKp44 (22) and mouse pDC–specific Siglec-H (23) signal through DAP12. In pDCs, triggering of these receptors initiates a signaling pathway involving spleen tyrosine kinase (SYK), Bruton's tyrosine kinase, B-cell linker protein, phospholipase Cγ 2 (PLCγ2), MEK-ERK, and induction of intracellular Ca^{2+} mobilization, similar to the pathway that occurs downstream of the BCR (18). Despite the similarity with BCR pathway, BDCA-2 signaling does not lead to the activation of the canonical NF-κB pathway monitored by the IκBα (16). Other RRs of pDC, such as dendritic cell immunoreceptor (DCIR, CLEC4A) (24), contain an ITIM motif. In spite of differences in ITAM or ITIM motifs, all these receptors inhibit TLR7/9 signaling (17, 18). Thus, the production of IFN-I in pDCs is controlled positively by immunogenic TLR7/9 and negatively by tolerogenic RRs.

Human cDCs, also called classical or myeloid dendritic cells, can be divided into at least two subsets. The more common mDC1s ($BDCA\text{-}1^+CD1c^+$), which produce inflammatory cytokines and chemokines, are major stimulators of T cells (25). The second subset, extremely rare mDC2s ($BDCA\text{-}3^+$ $XCR1^+Clec9A^+$) produce IL-12 and cross-present antigens for CD8 class I-restricted cytotoxic T lymphocytes (26, 27). cDCs detect invading microbes with the cell surface-expressed TLR1 and TLR2, which recognize peptidoglycan and lipoproteins, and endosomal compartment-localized TLR3, which recognizes double-stranded (ds)RNA, and TLR8, which recognizes single-stranded RNA (3–5). mDC2s are major producers of IFN-III, induced *via* dsRNA-sensing TLR3 pathway, independent of TLR7 (27). Immature cDCs sample the surrounding microenvironment for pathogens by numerous PRRs, including TLRs and ITAM-associated LLRs (6, 28, 29). Among ITAM-associated LLR expressed on cDCs, Dectin 2 (CLEC6A) and macrophage-inducible C-type lectin (Mincle, CLEC4E) associate with the ITAM-containing adaptor FcRγ, while myeloid DAP12-associated lectin-1 associates with the adaptor DAP12. Ligand binding to these LLRs leads in cDCs to phosphorylation of ITAM and recruitment of SYK like in pDCs (**Figure 1B**). However, in contrast to pDCs, recruitment of SYK in cDCs is followed by the formation of SYK–CARD9–BCL9–MALT1 complex, activation of the NF-κB subunit c-Rel, and production of pro-inflammatory cytokines (4, 6, 30). Surprisingly, this signaling pathway can result in the IRF5-mediated production of IFN-β without engagement of TLRs (31). Previous report of an alternative mechanism based on recognition of fungal infection by TLR7, independently of Dectin-1, makes induction of IFN-β in cDCs the matter of debate (32). While ligation of ITIM-associated LLR, such as myeloid C-type lectin-like receptor (MICL) or DCIR in cDCs inhibits TLR4 and TLR8 signaling (24, 33, 34), suppression of TLR signaling by ligation of ITAM-associated LLRs in cDCs has not been reported (28). These results together with the recent observation showing that Dectin-1-activated pDCs promote Th2-type T cell responses while Dectin-1-activated cDCs do the opposite, point to the importance of combination of PAMP, PRR, and the cell context in the regulation of adaptive immune responses by innate immunity (4, 35).

NEGATIVE SIGNALING BY ITAM-ASSOCIATED RECEPTORS IN MACROPHAGES

Results obtained during the two last decennia show that immune receptors associated with an ITAM can generate stimulatory or, paradoxically, inhibitory signals (36–44) (**Figures 2A,B**). These findings, obtained mostly in macrophages, provoked intense research into underlying mechanisms, as well as semantic debate (45). Inhibition can be readily explained by the paired co-clustering of ITIM-bearing receptors with the targeted ITAM-associated receptor, which brings them into close proximity for the consecutive inhibitory action of the tyrosine phosphatase SRC-homology-2 (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1) and SH2-domain-containing inositol phosphatase-1 (SHIP) (9, 24, 34, 42, 44) (**Figure 2B**). The work of several laboratories suggests that positive or negative control of immune responses, in the case of ITAM alone, is determined by avidity of ITAM-associated receptors to their ligands. The resulting "signal-switch hypothesis" is based on the observation that cross-linking by multimeric or high-avidity engagement of the ITAM-associated receptors leads to complete phosphorylation of ITAM tyrosine residues followed by the recruitment of SYK and to cell activation that synergizes IFN-I production, but inhibits cytokine signaling (8, 9, 39, 45, 46) (**Figure 2A**). In contrast, monovalent or low-avidity engagement of the ITAM-associated receptor results in monophosphorylation of the membrane-distal tyrosine (Y304) of ITAM allowing a transient recruitment and minimal activation of Syk (44) followed by actin depolymerization and translocation of protein or lipid phosphatases (SHP1, SHIP) instead of SYK to the ITAM in lipid rafts. Tyrosine phosphatases SHP1/2 and lipid phosphatase SHIP recruited to partially phosphorylated ITAM can inhibit TLR4 signaling by dephosphorylation of signaling intermediates, but concomitantly cell sensitivity to extracellular cytokines increases (**Figure 2B**). If a high-avidity stimulation of other receptors, such as FcγRs, FcεRI, the tumor necrosis factor receptors, chemokine CC-motif receptor 2, or TLRs, occurs in the proximity of a weak-avidity stimulation, the high-avidity-stimulated receptor is recruited toward the inhibitory SHP1 (9, 38, 42, 43, 46, 47). High-avidity signaling is deactivated by SHP1 in rafts and completed after internalization and segregation into polarized clusters called "inhibisomes," with SYK present at their periphery (9, 42).

MECHANISMS INHIBITING TLR SIGNALING IN MACROPHAGES

Spatiotemporal compartmentalization of inhibitory ITAM-containing receptors into lipid rafts is a key event in the triggering of several ITAM-mediated inhibitory signals. Thus, the presence of ITAMs in inhibisome rafts can be responsible for induction of a phosphatidylinositol 3-OH kinase (PI3K)- and PLCγ2-mediated imbalance characterized by accumulation of inositol-1,4,5-triphosphate ($InsP_3$) and low levels of diacylglycerol (**Figure 2B**). This imbalance results in triggering of constitutive calcium and MAPK signaling without phosphorylation of IκB at position Ser32 (47) and without activation of NF-κB, but it is sufficient to activate nuclear factor of activated T cells (8, 48). The importance of calcium signaling is highlighted by the finding that release of intracellular calcium activates the calcium-dependent phosphatase calcineurin, which is involved in the inhibition of TLR signaling by targeting the adaptor proteins MyD88 and TRIF (47).

A recent study showed that the TLR pathway in macrophages could be inhibited by another molecular mechanism, in which ITAM-associated low-avidity signaling inactivates MyD88 (49) (**Figure 3**). In this mechanism, SRC kinases-activated SYK phosphorylates Tyr227 on MyD88 and Tyr375 on TRIF, which function as substrates of the E3 ubiquitin ligase Cbl-b activated by β2-integrin CD11b (integrin α_M, Mac1). The role of β2-integrin in this interplay depends on the orientation of outside-in and inside-out signals. In inside-out signaling, TLR4 activates β2-integrin through PI3K and effector RapL by phosphorylation of the β2-integrin DAP12 adaptor ITAM, which attracts SYK (**Figure 3A**) and leads to separation of the cytoplasmic domains α and β of β2-integrin (**Figure 3B**). Then, in outside-in signaling, the activated β2-integrin engaged or not with a low-avidity ligand feeds back to inhibit TLR4 signaling by activation of SYK-mediated phosphorylation of MyD88 and TRIF, which are subsequently ubiquitinated by Cbl-b and degraded (49). Several reports have shown that upon low-avidity β2-integrin stimulation, Cbl-b

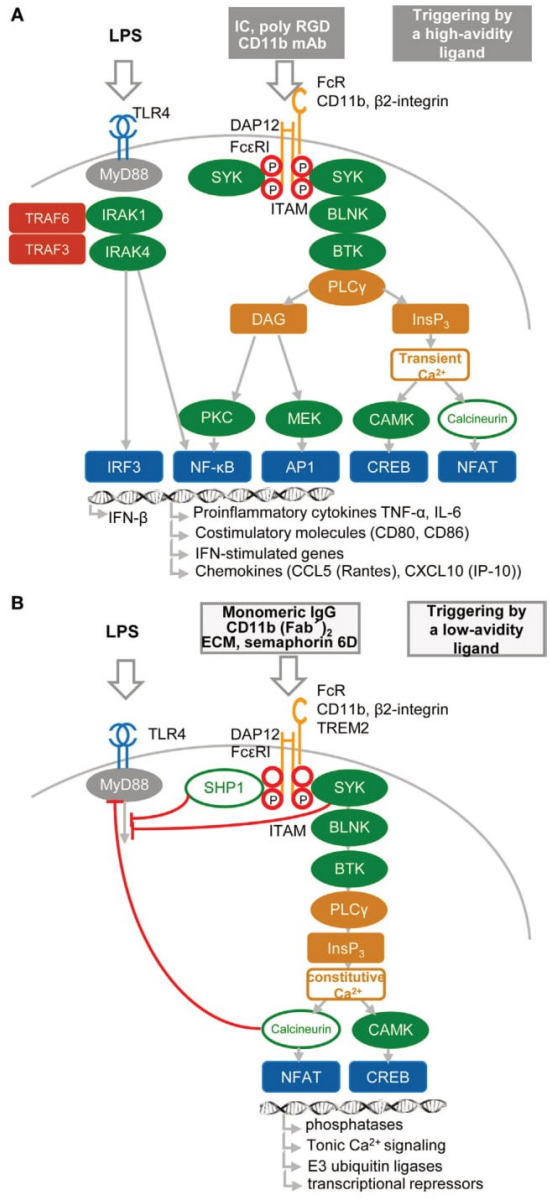

FIGURE 2 | Continued

FIGURE 2 | Continued
High- and low-avidity engagement of the immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors in macrophages. **(A)** High-avidity ligation of the ITAM-associated receptor results in synergy of ITAM and TLR4 signaling. High-avidity ligation of macrophage receptors, such as Fc receptors (FcRs) or β2-integrin [e.g., by cross-linking of FcR with immune complexes (IC), or β2-integrin with CD11b mAb or poly arginine–glycyl–aspartic acid (RGD) motifs (8)], induces activation signals that lead to phosphorylation of tyrosine residues within the DNAX activation protein 12 (DAP12) ITAM motif and to recruitment of spleen tyrosine kinase (SYK) kinase. DAP12 ITAM-mediated signaling involving SYK, B-cell linker protein (BLNK), Bruton's tyrosine kinase (BTK), and PLCγ proceeds as the signaling triggered by regulatory receptors in plasmacytoid DCs (**Figure 1B**). Ligation of TLR4 with its agonist (LPS) induces formation of a signalosome (from a set including IRAK1, IRAK4, TRAF3, and TRAF6), which activates IRF3 and production of IFN-β and pro-inflammatory cytokines (8, 9, 46). Tyrosine residues in ITAM motifs are shown by red circles. **(B)** Low-avidity ligation of the ITAM-associated receptor [e.g., by ligation of FcγR with monomeric IgG as exemplified by ligation of FcγRIIA with AT-10 $F(ab')_2$ (44), β2-integrin with CD11b $F(ab')_2$, or with Extracellular matrix (ECM) or triggering receptor expressed on myeloid cells 2 (TREM2) with semaphorin 6D] results in inhibition of TLR4 signaling. Low-avidity engagement of a high-affinity receptor results in recruitment of the SRC-homology-2 (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1), SHP2, and SH2-domain-containing inositol-5-phosphatase (SHIP) to the monophosphorylated membrane-distal tyrosine (Y304) of ITAM (44), shown by a letter P within a red circle. SHP1, SHP2, or SHIP can dephosphorylate TLR4 signaling intermediates. Low-avidity receptor ligation changes the balance between calcium and protein kinase C (PKC)-mediated pathways, leading to increased activity of calmodulin-dependent kinase (CAMK) and nuclear factor of activated T cells (NFAT) in the absence of NF-κB or mitogen-activated protein kinase activation.

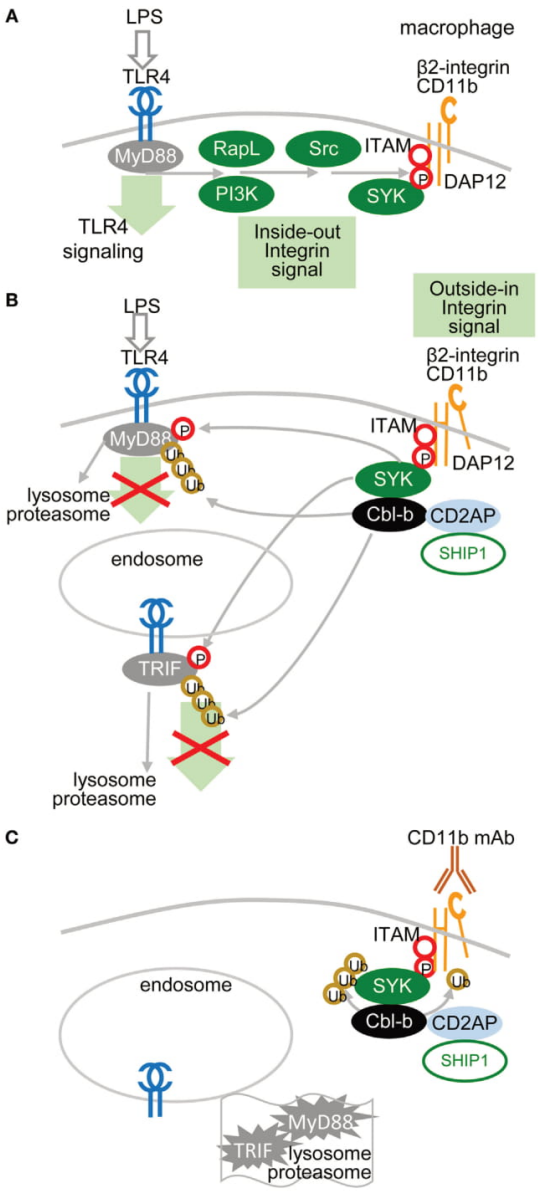

FIGURE 3 | Low-avidity signaling of β2-integrin regulates toll-like receptors (TLR) signaling in macrophages. (A) Inside-out signals are initiated by TLR4 activation through phosphatidylinositol 3-OH kinase (PI3K) and effector RapL β2-integrin by separation of the cytoplasmic domains of α- and β-integrin chains. **(B)** β2-integrin signals in a low-avidity (without even ligand binding) outside-in manner through ITAM-associated activation of the spleen tyrosine kinase (SYK) pathway, which induces phosphorylation of Tyr227 on MyD88 and of Tyr375 on TRIF. TLR4 signaling is regulated by Cbl-b-mediated degradation of MyD88 and TRIF. Skewed position of β2-integrin indicates conformational changes after separation of the cytoplasmic domains α and β. **(C)** Cbl-b associates with CD2-associated protein (CD2AP, CIN85) and enhances the ubiquitination and degradation of SYK and FcεRIγ. Ubiquitinated MyD88 and TRIF are degraded by the proteasome.

associates with CD2-associated protein (CIN85) and enhances the ubiquitination and degradation of SYK and FcεRIγ, resulting in inhibition of RRs (BCR-like) signaling (49–52) (**Figure 3C**). It has been reported that activation of SYK at the plasma membrane suppresses the TRAF6- and TAK1-mediated pro-inflammatory pathway and in contrast enhances the production of IFN-I *via* TBK-1 and IRF3 activation (53, 54). Thus, the global activation status of the target cell will be responsible for the outcome of TLR signaling.

In contrast to the well-established role of MAPK in activation of IFN-β production, several recent reports highlight suppressive aspects of MAPK signaling in myeloid cells (41, 43, 55–59). A central regulator that modulates repartition of MAPK signaling is Tumor Progression Lokus 2 (TPL-2 or Map3k8). TPL-2 down-modulates production of IFN-β and IL-12 (57) in macrophages, while it induces production of TNF-α (60) and IL-1β (61). It has been shown that activation of the MAPK (ERK) pathway by TPL-2 results, in macrophages but not in pDCs, in translocation of c-Fos into the nucleus and in inhibition of IFN-β gene transcription (57).

HIGH-AVIDITY ENGAGEMENT OF THE ITAM-ASSOCIATED RRs IN pDCs INHIBITS TLR7/9 SIGNALING

A cornerstone of the signal-switch hypothesis in macrophages is a direct relation between the avidity of the ITAM-associated receptor engagement and the intensity of IFN-I production (8, 9, 39, 45, 46). Surprisingly, in pDCs, the high-avidity cross-linking of BDCA-2 with mAb, as documented from protein tyrosine phosphorylation, activation of PLCγ, and intracellular Ca^{2+} release, results in the attenuation of TLR7/9-induced production of IFN-α and pro-inflammatory cytokines (17, 18) (**Figure 4**). As we and others demonstrated in pDCs, in contrast to macrophages, low-avidity engagement of BDCA-2 with monovalent anti-BDCA-2 Fab fragment, which does not induce any protein tyrosine phosphorylation in pDCs, fails to inhibit IFN-α production (62). As with BDCA-2, it is also the case that high-avidity engagement of the $FcεRIγ_{ITAM}$-associated receptor ILT7 or FcεRIα, or of the $DAP12_{ITAM}$-associated natural cytotoxicity receptor NKp44, is accompanied by protein tyrosine phosphorylation, calcium influx, and inhibition of IFN-I and pro-inflammatory cytokine production (19–22, 63). In addition to cross-linking with mAbs, high-avidity engagement of ILT7 with its natural ligand, bone marrow stromal cell antigen 2 (BST2, also called CD317, tetherin, or HM1.24) (19, 20, 63); of BDCA-2 with HIV-1 gp120 (64) or hepatitis C virus E2 glycoprotein (65); and of NKp44 with proliferating cell nuclear antigen also result in inhibition of IFN-I production (66).

The mechanism explaining inhibition of TLR7/9 signaling by high-avidity engagement of the ITAM-associated receptors in pDCs is not clear. The principal difference between pDCs and macrophages could reside in the localization and timing of the early steps of interaction of TLRs and ITAM-associated receptors. While in macrophages, the TLR4 and the ITAM-mediated β2-integrin signaling are concomitantly triggered from a close

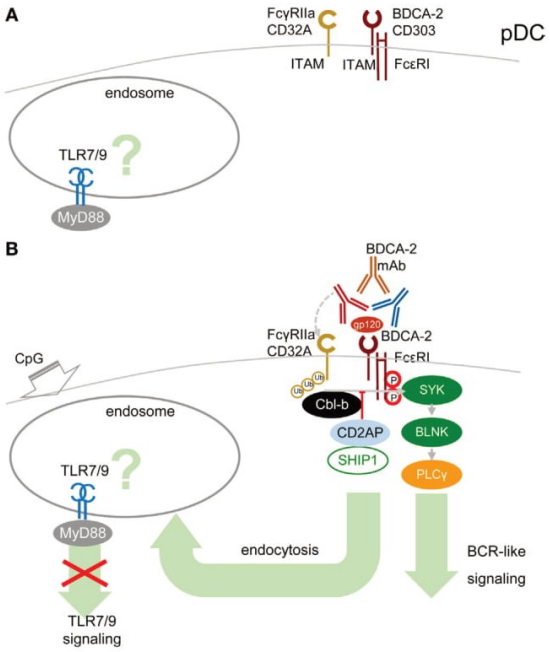

FIGURE 4 | High-avidity engagement of the immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors in plasmacytoid DCs (pDCs) inhibits TLR7/9 signaling. (A) In immature pDCs, blood dendritic cell antigen 2 (BDCA-2) and CD32a (FcγRIIa) are expressed on the cell surface. Homeostatic levels of TLR7/9 are present in endosomes. **(B)** BDCA-2 mAb or HIV-1 gp120/anti-gp120 Ab complex simultaneously engages BDCA-2 and CD32a, leading to internalization of both receptors. Crosslinking of BDCA-2-induces B-cell receptor (BCR)-like ITAM-mediated signaling. BDCA-2 cross-linking induces formation CD2AP/SHIP1 complex, which inhibits the Cbl-b-induced ubiquitination of spleen tyrosine kinase (SYK), FcεRIγ, and FcγRIIa and maintains the expression levels of SYK and FcεRIγ.

vicinity in the lipid raft (**Figure 3**), in pDCs, the TLR7/9 signaling is triggered from an endosome, whereas ITAM-mediated RRs signaling is triggered from the plasma membrane, with an unknown delay. Earlier findings suggested that in unstimulated pDCs, TLR7 and TLR9 reside in the endoplasmic reticulum and are delivered to the endolysosomal compartment only after uptake of RNA or DNA ligands to endosomes (10, 67, 68). However, more recent studies have demonstrated a steady-state flow of TLR9 from endoplasmic reticulum to endolysosomes, where TLR9 is present in the mature, cathepsin-cleaved form (69, 70) (**Figure 4A**). Most of these studies were performed in mouse bone marrow-derived macrophages, and parallel experiments in B cells showed that TLR9 trafficking is cell-context-dependent, making an actual localization of TLR7/9 in pDCs a matter of debate (69, 70). As with TLR9 trafficking, trafficking of RRs in pDCs remains elusive. Formation of the antibody–BDCA2 receptor complex (17), which colocalizes with EEA1 in early endosomes 5–10 min after crosslinking of BDCA2, was demonstrated in several laboratories (71, 72). Results from our laboratory have shown that 2 min after crosslinking of BDCA-2, SYK is phosphorylated, without assigning the phosphorylation to plasma membrane or endosome (73) (**Figure 4B**). Whether TLR7/9 and BDCA-2 co-localize in endolysosomes (71, 72), and at what level, remains to be determined.

As in macrophages, ubiquitination of MyD88, SYK, and FcεRIγ could play a crucial role in the outcome of TLR7/9 and RRs signaling in pDCs. Ubiquitination of these molecules depends on the cellular context. It has been shown that upon BDCA2 cross-linking in human pDCs, CD2AP forms a complex with SHIP1 and Cbl-b with reduced Cbl-E3 ubiquitin ligase activity in comparison with CD2AP- or SHIP1-knocked-down pDCs (3, 74) (**Figure 4B**). The CD2AP/SHIP1/Cbl complex is then recruited to the plasma membrane, where it co-localizes with cross-linked BDCA2/FcεR1γ complex. Inhibition of the Cbl-b-induced ubiquitination and degradation of FcεR1γ and SYK by the CD2AP/SHIP1 complex results in upregulation of BCR-like signaling and inhibition of TLR7/9 signaling. Although these results were not reproduced in mouse pDCs (75), differences between the triggering of TLR and ITAM signaling in macrophages and in pDCs (**Figures 1** and **2**) might be responsible for preferential ubiquitination of MyD88, SYK, and FcεRIγ in these cells.

In addition to ITAM-associated RRs, pDCs express an ITAM-associated FcR, FcγRIIa (CD32a), which is responsible for uptake and delivery of systemic lupus erythematosus (SLE) immune complexes (IC) in the endosomal compartment called the IFN signaling compartment, from where they trigger TLR9 signaling followed by massive IFN-I production (72, 76). ITAM-associated CD32a and ITIM-associated CD32b are the only FcRs on pDCs (72, 76). Surprisingly, BDCA-2 mAb simultaneously ligates BDCA-2 with $F(ab')_2$ and CD32a with the Fc region of the same single BDCA-2 mAb molecule, leading to the concurrent internalization of BDCA-2 and CD32a (**Figure 4B**). Simultaneous engagement of BDCA-2 and CD32a with complete BDCA-2 mAb, in contrast to ligation with $F(ab')_2$, synergizes inhibition of TLR9 signaling triggered by FcR-dependent SLE IC. However, simultaneous engagement does not potentiate inhibition of TLR7/9 signaling triggered by FcR-independent agonists CpG-A, CpG-B, and R848 (72). Thus, the latter results do not lend any support to the hypothesis that the simultaneous ligation of BDCA-2 and CD32a with a single mAb molecule, creating a trimolecular complex, would be responsible for BDCA-2 mAb-mediated inhibition of TLR9 signaling and IFN-I production (9, 38, 46, 47, 72). The synergistic effect of simultaneous engagement of CD32a and BDCA-2 highlights the therapeutic potential of these mAbs for inhibition of IFN-I production and for treatment of autoimmune diseases, such as SLE. Next, experiments can show whether natural ligands of RRs, such as BST2, HIV-1 gp120, hepatitis C virus E2 glycoprotein, or their complexes with antibody could cross-link BDCA-2/ILT7 and CD32a and inhibit TLR7/9 signaling (**Figure 4B**).

TOLEROGENIC EFFECT OF THE HIGH-AVIDITY ENGAGEMENT OF ITAM-ASSOCIATED RECEPTORS IN pDCs

Antigen targeted to pDCs by means of BDCA-2 mAb is rapidly endocytosed and traffics *via* early endosomes to MHC-enriched

endosomes independently of TLR7/9 stimulation (71). However, the next steps of antigen presentation, including restimulation of antigen-specific $CD4^+$ effector memory T helper cells, are dependent on TLR7/9 stimulation of pDCs, which is inhibited by BDCA-2 cross-linking. Mature pDCs are characterized by the upregulation of CD40 and co-stimulatory molecules, including CD80 and CD86. Recent results have shown that BDCA-2 mAb cross-linking inhibits CpG-A and CpG-B-induced upregulation of co-stimulatory molecules CD40 and CD86 (62, 77–79). In contrast, CD40L-stimulated upregulation of CD86 in pDCs is unaffected by BDCA-2 cross-linking. These results suggest that BDCA-2 signaling interferes with TLR9 signaling in pDCs but probably not with T-cell-dependent pDC activation *via* CD40-ligand (62). Actually, pDCs efficiently cross-present exogenous antigens to $CD8^+$ T cells (80). Also, BDCA-2 agonist HIV-1 gp 120, but not the natural agonist of ILT7, BST2, suppressed TLR9-mediated expression of co-stimulatory molecules CD80 and CD86 by pDCs (19, 64).

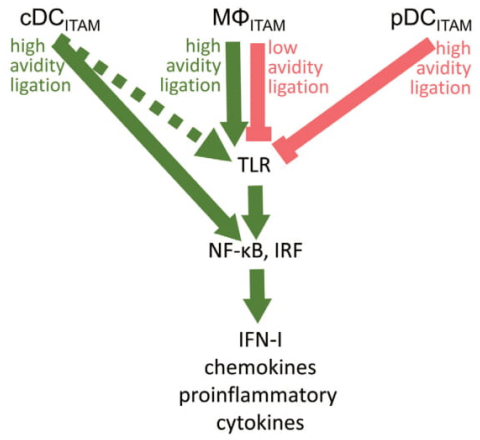

FIGURE 5 | Cross talk between immunoreceptor tyrosine-based activation motif (ITAM)-associated receptor signaling and toll-like receptor (TLR) pathways in conventional dendritic cells (cDCs), MΦ, and plasmacytoid DCs (pDCs): an ITAM-centric view. ITAM-mediated activation pathways are shown by green arrows; ITAM-mediated inhibitory pathways are shown by red lines. Positive or negative control of immune responses in macrophages (MΦ) is determined by avidity of ITAM-associated receptors to their ligands. Production of interferons (IFNs)-I is facilitated by interferon-regulatory factor 3 (IRF3) in macrophages, by IRF5 in cDCs, and by IRF7 in pDCs. In cDCs, ITAM-associated receptor signaling can result in the IRF5-mediated production of IFN-β without engagement of TLRs (31). Alternative pathway in cDCs (32) is shown by dotted arrow.

The capacity of pDCs to produce IFN-I and their central role at the interface of innate and adaptive immunity could make them important actors in antitumor immunity (81). However, recent evidence suggests that tumor-associated (TA) pDCs recruited in breast and ovarian tumors are dysfunctional and their presence in these tumors is a negative prognostic factor for overall survival (82–84). This dysfunctionality is characterized by impairment of their IFN-I secretion and by strong expression of ICOS ligand, which leads to induction of immunosuppressive regulatory T cells (Treg) and priming of IL-10-secreting $CD4^+$ T cells (82, 83). Apart from tumor-derived soluble immunosuppressive factors, such as TNF-α and TGF-β (85), recent data suggest that TA-pDC impairment could also result from the interaction of ITAM-associated RRs of pDCs with their ligands expressed on cancer cells, such as BST2 (20). Recent data from the C. Caux laboratory have shown that mAbs against ICOS inhibit TA-Treg expansion and IL-10 secretion, demonstrating a pivotal role of TA-pDCs in the immunosuppressive mechanism (82, 83). Collectively, these results indicate that a tumor microenvironment induces a tolerogenic character in pDCs.

TABLE 1 | Cross talk between immunoreceptor tyrosine-based activation motif (ITAM)-signaling and toll-like receptors (TLR) pathways in macrophages and plasmacytoid DCs (pDCs).[a]

	TLR		ITAM-coupled receptors				ITAM/TLR cross talk	
	Receptor	Ligand	Receptor	Adaptor	Ligand		High-avidity ligation	Low-avidity ligation
					High avidity	Low avidity		
Macrophage	TLR4	LPS	β_2-Integrin	DAP12	Fibrinogen	ECM	• Synergizes TLR signaling • Inhibits cytokine signaling • Activation	• Inhibits TLR signaling • Enhances cytokine signaling • Homeostasis
			FcγRI	FcRγ	IC/RF	Monomeric IgG or IgA, IVIg, mAb F(ab′)$_2$		
			FcγRIIA	–	IC/RF			
			FcαR	FcRγ	IC/RF			
			FcεRI	FcRγ	IC/RF			
			TREM2	DAP12	poly RGD	Semaphorin 6D		
pDCs	TLR7	ssRNA Resiquimod	BDCA-2	FcεR1γ	HIV gp120, HCV E2; mAb	mAb Fab, F(ab′)$_2$	• Inhibits TLR signaling • Inhibits pDC maturation and T cell stimulation • Homeostasis/anergy	No/unknown effect
			ILT7	FcεR1γ	BST2			
			FcεRIα	FcεR1γ	IgE			
			NKp44	DAP12	PCNA			
	TLR9	CpG ODNs	Siglec-H	DAP12	Sialic acid			
			FcγRIIA	–	IC			

[a]*mAb, mAb Fab, and mAb F(ab′)$_2$ are related to the respective receptor.*

IC, immune complexes; RF, rheumatoid factor; IVIg, intravenous immunoglobulin; TREM2, triggering receptor expressed on myeloid cells 2; ECM, extracellular matrix; RGD, arginine–glycyl–aspartic acid motif; DAP12, DNAX activation protein 12; BDCA-2, blood dendritic cell antigen 2; ILT7, immunoglobulin-like transcript; PCNA, proliferating cell nuclear antigen.

CONCLUDING REMARKS

Fifteen years after the discovery of the inhibitory role of BDCA-2 in IFN-I production in pDCs (17), its molecular mechanism remains elusive. The signal-switch hypothesis had a seminal role in the understanding of cross-regulation of cytokine- and TLR-signaling pathways in macrophages (8, 9, 28, 39, 58). However, further studies showed that the ITAM-signaling pathway may be regulated in a special way in human pDCs (11, 18–20). While the high-avidity engagement of ITAM-associated receptors in macrophages leads to potentiation of TLR signaling, it results in the attenuation of TLR-induced IFN-I production in pDCs (**Figure 5**; **Table 1**). Surprisingly, few data are available on the interplay of ITAM-associated receptors and TLRs in cDCs. Cellular context, spatiotemporal differences, and different functions of ITAM-associated receptors could be responsible for the different interplay of ITAM and TLR pathways in pDCs, cDCs, and macrophages.

Published data suggest that ITAM-associated receptors play different roles in pDCs, cDCs, and in macrophages. Under homeostatic conditions in macrophages, the ITAM-associated receptors enable a fine-tuning of immune responses, including inhibition of IFN-I production and high sensitivity to extracellular cytokines. In an infection setting, ITAM-associated receptors in macrophages switch to signaling for robust production of cytokines including IFN-I, to cell activation and to low sensitivity to extracellular cytokines. In cDCs, ligation of ITAM-associated receptors leads to rapid activation of NF-κB and massive production of cytokines, which can occur without engagement of TLR. In contrast, the major role of ITAM-associated RRs in pDCs is related to inhibition of cytokine production and reestablishment of a tolerogenic state following pDC activation. Limitation of the ITAM-associated RR signaling in pDCs to high-avidity engagement could be related to a low homeostatic level of TLR7/9 in endosomes in immature pDCs under physiological conditions (10, 70). The low homeostatic level of TLR7/9 in endosomes in immature pDCs could reduce the risk of undesirable triggering of IFN-I signaling to the same extent as that of the inhibition of IFN-I induced in macrophages by tonic ITAM signaling. Also, simultaneous engagement of BDCA-2 and CD32a leading to the internalization of both receptors is consistent with the tolerogenic role of BDCA-2. Differential effects of ITAM-mediated signaling in pDCs and macrophages would promote a coordinated cellular response to infection and inflammation.

Interaction of the TLR pathway and ITAM signaling in pDCs plays an important role in control of the innate immune responses in viral infections (1, 64, 65), cancer proliferation (20, 83, 84), and autoimmune diseases (54, 86). Understanding the ITAM/TLR-signaling network in pDCs may serve as an effective means for positive and negative control of pDC activation. Progress in understanding these interactions paves the way for the development of compounds to control activation of pDCs. Pharmacologic targeting of TLR and ITAM signaling is thus an attractive new therapeutic approach for treatment of chronic infections, cancer, and autoimmune and inflammatory diseases.

AUTHOR CONTRIBUTIONS

The work was written by IH and NB-V with substantial contributions of VJ and RS to the conception, drafting, and revising the work for important intellectual content. All authors gave final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The MEK1/2-ERK Pathway Inhibits Type I IFN Production in Plasmacytoid Dendritic Cells

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Recent studies have reported that the crosslinking of regulatory receptors (RRs), such as blood dendritic cell antigen 2 (BDCA-2) (CD303) or ILT7 (CD85g), of plasmacytoid dendritic cells (pDCs) efficiently suppresses the production of type I interferons (IFN-I, α/β/ω) and other cytokines in response to toll-like receptor 7 and 9 (TLR7/9) ligands. The exact mechanism of how this B cell receptor (BCR)-like signaling blocks TLR7/9-mediated IFN-I production is unknown. Here, we stimulated BCR-like signaling by ligation of RRs with BDCA-2 and ILT7 mAbs, hepatitis C virus particles, or BST2 expressing cells. We compared BCR-like signaling in proliferating pDC cell line GEN2.2 and in primary pDCs from healthy donors, and addressed the question of whether pharmacological targeting of BCR-like signaling can antagonize RR-induced pDC inhibition. To this end, we tested the TLR9-mediated production of IFN-I and proinflammatory cytokines in pDCs exposed to a panel of inhibitors of signaling molecules involved in BCR-like, MAPK, NF-κB, and calcium signaling pathways. We found that MEK1/2 inhibitors, PD0325901 and U0126 potentiated TLR9-mediated production of IFN-I in GEN2.2 cells. More importantly, MEK1/2 inhibitors significantly increased the TLR9-mediated IFN-I production blocked in both GEN2.2 cells and primary pDCs upon stimulation of BCR-like or phorbol 12-myristate 13-acetate-induced protein kinase C (PKC) signaling. Triggering of BCR-like and PKC signaling in pDCs resulted in an upregulation of the expression and phoshorylation of c-FOS, a downstream gene product of the MEK1/2-ERK pathway. We found that the total level of c-FOS was higher in proliferating GEN2.2 cells than in the resting primary pDCs. The PD0325901-facilitated restoration of the TLR9-mediated IFN-I production correlated with the abrogation of MEK1/2-ERK-c-FOS signaling. These results indicate that the MEK1/2-ERK pathway inhibits TLR9-mediated type I IFN production in pDCs and that pharmacological targeting of MEK1/2-ERK signaling could be a strategy to overcome immunotolerance of pDCs and re-establish their immunogenic activity.

Keywords: plasmacytoid dendritic cells, toll-like receptors 7 and 9 (TLR7/9), B cell-like receptor signaling, regulatory receptors, blood dendritic cell antigen 2, MEK1/2, c-FOS, type I interferon

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INTRODUCTION

Plasmacytoid dendritic cells (pDCs) are a highly specialized subset of dendritic cells that play a central role at the interface of innate and adaptive immunity. They are important actors in antiviral and antitumor immunity, but also potent inducers of autoimmune diseases (1–6). They sense viruses by endosomal toll-like receptors 7 and 9 (TLR7/9), recognizing ssRNA or CpG containing DNA. TLR signaling leads to the secretion of proinflammatory cytokines and chemokines, such as interleukin 1, tumor necrosis factor α (TNF-α), IL-6, IL-8, and most importantly type I IFNs (IFN-I, α/β/ω) (7–10).

In addition to TLR7/9, pDCs express multiple specific receptors that facilitate antigen capture and presentation and, moreover, regulate pDC function, preventing thus abnormal immune responses. These regulatory receptors (RRs), include Fc receptors and lectin-like receptors (11, 12), which signal through the B cell receptor (BCR)-like pathway involving spleen tyrosine kinase (SYK) associated with the immunoreceptor tyrosine-based activation motif-containing adapter of RR, Bruton's tyrosine kinase, B-cell linker protein, phospholipase Cγ 2, MEK1/2-ERK, and induction of intracellular Ca2+ mobilization (8, 9, 12). Among these RRs, blood dendritic cell antigen 2 (BDCA-2, CD303, CLEC4C) is an lectin-like receptor (13), while immunoglobulin-like transcript (ILT7, CD85g) binds to and can be activated by bone marrow stromal cell antigen 2 (BST2, CD317, tetherin, HM1.24) protein, the expression of which is found on cells pre-exposed to IFN-I or on the surface of human cancer cells (14). Signaling *via* pDC RRs attenuates TLR-induced production of IFN-I and proinflammatory cytokines by an unknown mechanism (8–13, 15, 16). This physiological feedback mechanism of IFN control is hijacked in the pathogenesis of several chronic viral infections and cancers, leading to immune tolerance (10, 17–19). We have recently shown that hepatitis C virus (HCV) particles inhibit the production of IFN-α *via* the binding of E2 glycoprotein to RRs BDCA-2 and DCIR (dendritic cell immunoreceptor) and induce a rapid phosphorylation of AKT and ERK, in a manner similar to the cross-linking of BDCA-2 or DCIR (10, 17, 19).

Here, we addressed the question of whether specific pharmacological targeting of BCR-like signaling can restore functionality to pDCs abrogated by ligation of RRs, and what the underlying mechanism of this abrogation is. In our previous work, we demonstrated that a highly specific inhibitor of SYK blocks both BCR-like and TLR7/9 signaling and, therefore, it is not compatible with restoration of pDC function (15). In this study, we have tested the effects of inhibitors of c-Jun N-terminal kinase (JNK), MEK1/2 kinase, p38 kinase, and calcium-dependent phosphatase calcineurin, acting through a BCR-like signaling pathway, and of NF-κB activating TANK binding kinase 1 (TBK1) on the IFN-I production in pDCs exposed to a TLR9 agonist. Surprisingly, we found that inhibitors of MEK1/2 potentiated IFN-I and IL-6 production in pDC cell line GEN2.2, but not in primary pDCs stimulated by the TLR9 agonist. More importantly, inhibitors of MEK1/2 significantly increased TLR9-mediated production of IFN-I that had been blocked in both GEN2.2 cells and primary pDCs by ligation of RRs with BDCA-2 and ILT7 mAbs, or HCV particles, or with BST2 expressing cells. Moreover, the restauration of IFN-I production by MEK1/2 inhibitor was observed when TLR9 signaling had been blocked by phorbol 12-myristate 13-acetate (PMA), an agonist of protein kinase C (PKC), which stimulates MEK1/2-ERK signaling.

Furthermore, our results show that BCR-like and PKC signaling induced in pDCs the expression and phoshorylation of c-FOS, a downstream gene product of the MEK1/2-ERK pathway. c-FOS is known to associate with c-JUN to form activator protein 1 (AP-1) transcription factor and to exert within the cell a pleiotropic effect, including cell differentiation, proliferation, apoptosis, and the immune response (20–23). While a previous study reported that the c-FOS induced by tumor progression locus 2 (TPL-2) inhibits TLR9-mediated production of IFN-I in mouse macrophages and myeloid DCs, but not in pDCs (24), we show that MEK1/2-ERK-induced c-FOS was involved in the inhibition of TLR9-mediated production of IFN-I in human pDCs. Our results suggest that the MEK1/2-ERK-dependent expression and phosphorylation of c-FOS exerts an intrinsic block of TLR9-mediated production of type I IFN. Pharmacological targeting of MEK1/2-ERK signaling could be a strategy to overcome immunotolerance of pDCs and re-establish their immunogenic activity.

RESULTS

MEK1/2 Inhibitor Potentiates CpG-A-Induced Production of IFN-α in pDC Cell Line GEN2.2

In order to restore TLR7/9-mediated production of IFN-I blocked by ligation of RRs, we first searched for an inhibitor of BCR signaling that does not inhibit signaling triggered by TLR7/9 agonists. To this end, we selected a panel of kinase inhibitors involved in BCR-like, MAPK, NF-κB, and calcium signaling, and control inhibitors of TLR7/9 signaling, and tested their effect on the production of IFN-α in a pDC cell line GEN2.2 exposed to TLR9 agonist CpG-A (**Figures 1A,B**; Figure S1 in Supplementary Material). To facilitate biochemical analyses of cell signaling, which is still difficult to perform in rare and *in vitro* short living human primary pDCs, we performed our studies in human pDC line GEN2.2, which shares the key features of human primary pDCs (15, 25–30).

While inhibitors of JNK (SP600125), TBK1 (BX795), NF-κB (Bay11-7082), p38 MAPK (SB253080), and calcineurin (FK506) inhibited dramatically IFN-α production, MEK1/2 inhibitor PD032590 significantly increased IFN-α production ($p = 0.0022$, **Figure 1B**). In repeated independent experiments ($N = 34$), production of IFN-α in CpG-A-stimulated GEN2.2 cells increased 2.55 ± 0.63 times (mean ± SEM, $p < 0.0001$),

Abbreviations: AP-1, activator protein 1; BLNK, B-cell linker protein; BCR, B cell receptor; BDCA-2, blood dendritic cell antigen 2; BST2, bone marrow stromal cell antigen 2; BTK, Bruton's tyrosine kinase; FcRs, Fc receptors; geq, genome equivalent; HCV, hepatitis C virus; ITAM, immunoreceptor tyrosine-based activation motif; IL-1, interleukin 1; IFN-I α/β/ω, type I interferons; PMA, phorbol myristoyl acetate; PLCγ2, phospholipase Cγ 2; PKC, protein kinase C; RRs, regulatory receptors; SRE, serum response element; SYK, spleen tyrosine kinase; TNF-α, tumor necrosis factor α; TPL-2, tumor progression locus 2; TBK1, TANK binding kinase 1.

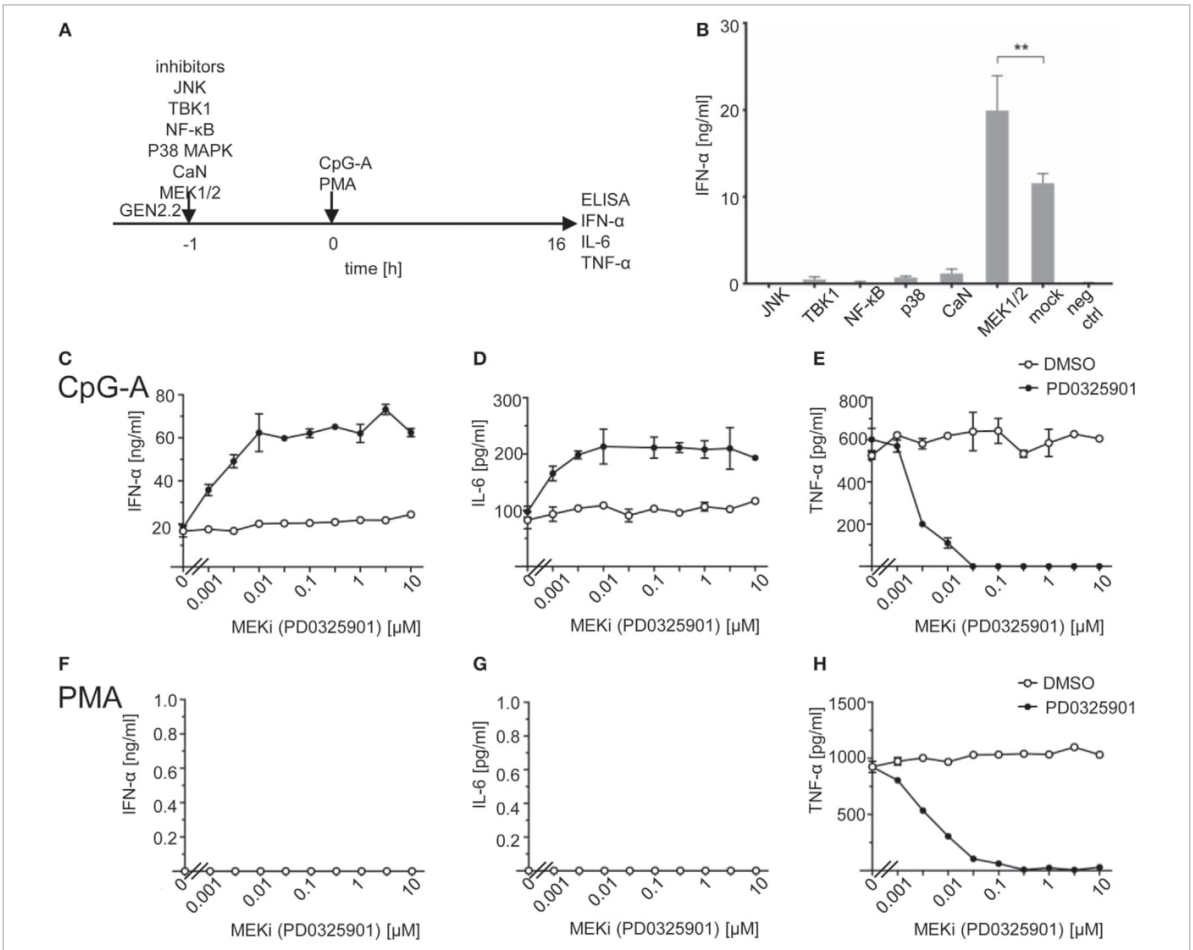

FIGURE 1 | Effect of MEK1/2 inhibitor PD0325901 on cytokine production in CpG-A and phorbol myristoyl acetate (PMA)-stimulated GEN2.2 cells. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were exposed or not to inhibitors of Jun N-terminal kinase (JNK), TANK binding kinase 1 (TBK1), NF-κB, p38 MAPK, calcineurin, or MEK1/2 for 1 h and then stimulated with CpG-A at 4 μg/ml. The concentration of IFN-α, IL-6, and tumor necrosis factor α (TNF-α) in the cell-free supernatant was determined by ELISA after a 16 h 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.

from 18.4 ± 1.4 ng/ml in the absence of MEK1/2 inhibitor to 44.2 ± 2.7 ng/ml in the culture pretreated with 1 μM PD0325901 (Figure S2 in Supplementary Material). In spite of the variability of IFN-α production in CpG-A-stimulated GEN2.2 cells, the ratio of IFN-α production in GEN2.2 cells cultured in the presence and in the absence of PD0325901 was highly reproducible. The same results were obtained with MEK1/2 inhibitor U0126 (data not shown). We found that in addition to IFN-α also IL-6 production in CpG-A-stimulated GEN2.2 cells was synergized by MEK1/2 inhibitor PD0325901 (**Figures 1C,D**), whereas production of TNF-α was inhibited (**Figure 1E**), suggesting that the MEK1/2-ERK pathway positively regulates TNF-α expression or secretion (31). The strongest synergistic effects on IFN-α production (synergistic index >3) were observed for combinations of ≥0.01 μM PD0325901 and 4 μg/ml CpG-A. Synergistic effects of these combinations were also demonstrated for the production of IL-6 (synergistic index >2). In contrast to the synergistic effect observed with ≥0.01 μM PD0325901, the combination of 0.001 μM PD0325901 with 4 μg/ml CpG-A had only an additive effect on the production of IL-6 (**Figure 1D**). In the control experiment, PMA-induced the production of TNF-α (but not that of IFN-α and IL-6), which was strongly inhibited by

PD0325901 (**Figures 1F–H**). Collectively, these results show that the CpG-A-induced TLR9-mediated production of IFN-α and IL-6 are potentiated by MEK1/2 inhibitor PD0325901.

MEK1/2 Inhibitor Potentiates Herpesvirus- and CpG-B-Induced Production of IFN-α in pDC Cell Line GEN2.2

CpG-A is a synthetic mimic of an unmethylated CpG-rich dsDNA of bacteria and viruses. Therefore, we tested whether production of IFN-α in GEN2.2 cells stimulated with natural TLR9 agonists, herpes simplex virus type 1 (HSV-1), and human cytomegalovirus (HCMV) could be potentiated with PD0325901 (**Figures 2A,B**). Our results show that PD0325901 significantly potentiated production of IFN-α in GEN2.2 cells exposed to HSV-1 (2.14-fold, $N = 3$, $p = 0.0022$), or HCMV (1.98-fold, $N = 3$, $p = 0.0022$).

While aggregating CpG-A is transported to the interferon-regulatory factor 7 endosomes, where activates production of IFN-I, monomeric CpG-B is transferred to the NF-κB endosomes, which leads to maturation of pDCs, formation of pro-inflammatory cytokines and only a limited production of IFN-α (7–10). PD0325901 significantly potentiated production of IFN-α in CpG-B-stimulated GEN2.2 cells (1.43-fold, $N = 7$, $p = 0.007$), although less strongly than in CpG-A-stimulated cells (**Figure 2C**). Taken together, MEK1/2 inhibitor PD0325901 potentiated production of IFN-α in pDC cell line GEN2.2 stimulated with synthetic TLR9 agonists CpG-A and CpG-B, and natural agonists HSV-1 and HCMV.

MEK1/2 Inhibitors Partially Restore TLR9-Mediated IFN-α Production Blocked by Ligation of RRs with BDCA-2 and ILT7 mAbs

Subsequently, with respect to the ability of PD0325901 to synergize TLR7/9-mediated IFN-α production, we investigated the capacity of PD0325901 to reverse the inhibitory effect of the ligation of RRs on TLR9-mediated IFN-α production. We exposed PD0325901-pretreated GEN2.2 cells and primary pDCs to 5 μg/ml of BDCA-2 mAb and subsequently to TLR9 agonist CpG-A (**Figure 3A**). In the absence of the MEK1/2 inhibitor, the production of IFN-α induced in GEN2.2 cells by CpG-A was suppressed by BDCA-2 mAb to 13% ($p = 0.0006$, **Figure 3B**). As already shown in **Figure 1C**, PD0325901 significantly potentiated CpG-A-induced production of IFN-α in GEN2.2 cells (3.8-fold, $N = 6$, $p = 0.0022$, **Figures 3B,C**). As expected, PD0325901 potentiated the production of IFN-α inhibited in GEN2.2 cells by BDCA-2 mAb. This partial restoration of IFN-α production in GEN2.2 cells was highlighted after standardization to the quantity of IFN-α produced in the absence of PD0325901 (7.3-fold, $p = 0.0022$, **Figure 3C**).

As in GEN2.2 cells, 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 3D**). The major difference observed in primary pDCs compared to GEN2.2 cells consisted in the lack of the potentiation of CpG-A-induced production of IFN-α by PD0325901 in the absence of BDCA-2 mAb (**Figures 3B–E**). In contrast, a similar restoration effect to the one in GEN2.2 was observed in primary pDCs

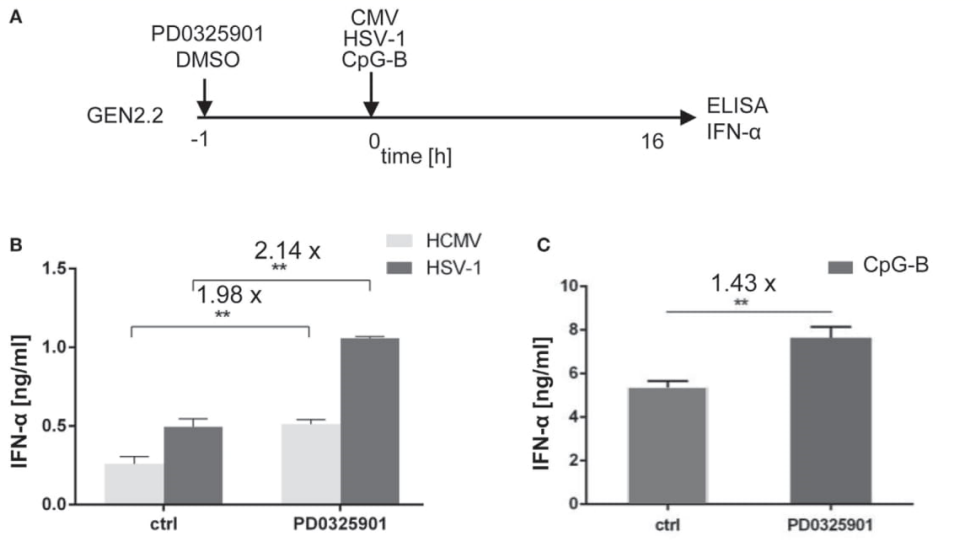

FIGURE 2 | Effect of MEK1/2 inhibitor PD0325901 on the potentiation of IFN-α production stimulated with HSV-1, human cytomegalovirus (HCMV), or CpG-B. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated with the MEK1/2 inhibitor PD0325901 (1 μM) for 1 h before stimulation with HSV-1 or HCMV at the MOI of 10 $TCID_{50}$ per cell, or with 4 μg/ml CpG-B. After a 16 h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. $N = 3$, **, $p < 0.01$; two-tailed Student's *t*-test. **(B)** The production of IFN-α by GEN2.2 cells stimulated with HSV-1 or HCMV in the presence or absence of PD0325901. **(C)** The production of IFN-α by GEN2.2 cells stimulated with CpG-B in the presence or absence of PD0325901. The data show mean and SEM of three independent experiments. $N = 7$, **, $p < 0.01$; two-tailed Mann–Whitney test.

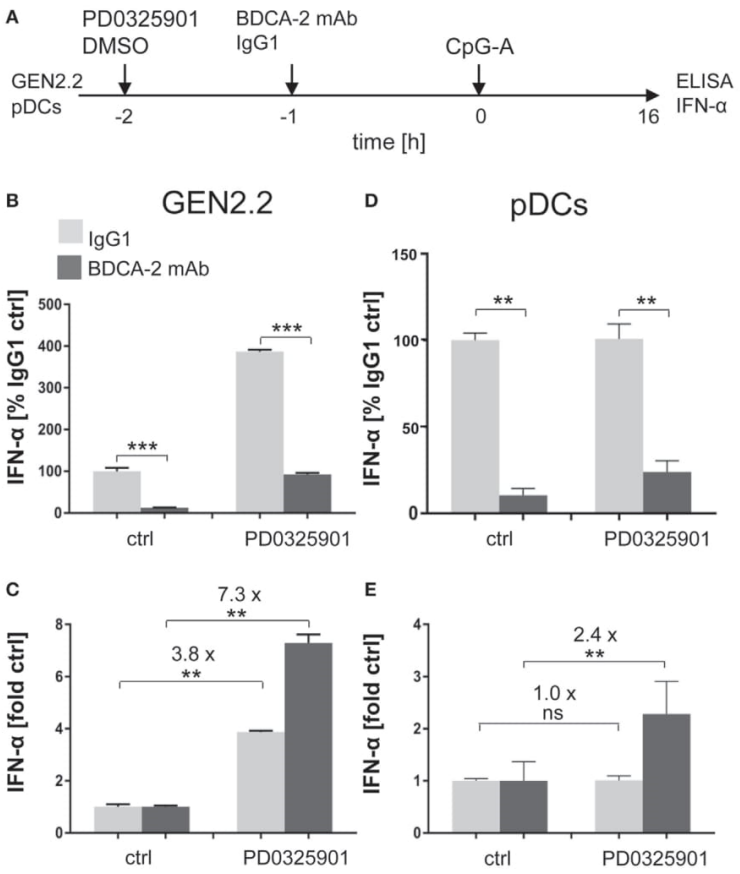

FIGURE 3 | Effect of MEK1/2 inhibitor PD0325901 on the blockade of IFN-α production by ligation of regulatory receptors of GEN2.2 cells or primary plasmacytoid dendritic cells (pDCs) with blood dendritic cell antigen 2 (BDCA-2) mAb. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells or primary pDCs were incubated with the MEK1/2 inhibitor 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,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 ± SEM of **(B,C)** six independent experiments with GEN2.2 cells, **, $p < 0.01$; ***, $p < 0.001$; 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.

exposed to PD0325901 prior to BDCA-2 mAb (**Figures 3D,E**). PD0325901 significantly restored the production of IFN-α inhibited by BDCA-2 mAb (2.4-fold, $p = 0.0039$, **Figure 3E**). A similar restoration effect was observed with PD0325901 at 10 nM concentration (Figure S3 in Supplementary Material) and with MEK1/2 inhibitor U0126 using ILT7 mAb for crosslinking RR (Figure S4 in Supplementary Material). In conclusion, these results show that MEK1/2 inhibitors significantly increased the TLR9-mediated IFN-I production blocked by ligation of RRs.

MEK1/2 Inhibitor Restores TLR7/9-Mediated IFN-α Production Blocked by HCV Virions

We and others reported that some viruses, such as HCV (19, 32), HBV (18), or HIV (17), interact *via* their envelope glycoproteins with RR BDCA-2 expressed on pDCs, and activate the BCR-like pathway leading to the inhibition of IFN-α production. We tested whether MEK1/2 inhibitor PD0325901 restores IFN-α production in pDC cell line GEN2.2 (**Figures 4A,B**) and in primary pDCs (**Figures 4A,C**) stimulated with CpG-A, and in parallel exposed to HCV particles (10 HCV geq/cell). We confirmed that in the absence of MEK1/2 inhibitor, HCV virions inhibited IFN-α production in both cell types, to 35% in GEN2.2 cells (**Figure 4B**) and to 34% in primary pDCs (**Figure 4C**) (19, 33). We observed that the treatment with PD0325901 significantly restored CpG-A-stimulated production of IFN-α inhibited by HCV virions in GEN2.2 cells (4.2-fold, $p = 0.025$, **Figure 4B**) and in primary pDCs (3.2-fold, $p = 0.0059$, **Figure 4C**), in a more robust way than that observed with BDCA-2 mAb (**Figure 3**). Collectively, pharmacological targeting of MEK1/2-ERK abrogates the HCV suppression of IFN-α production.

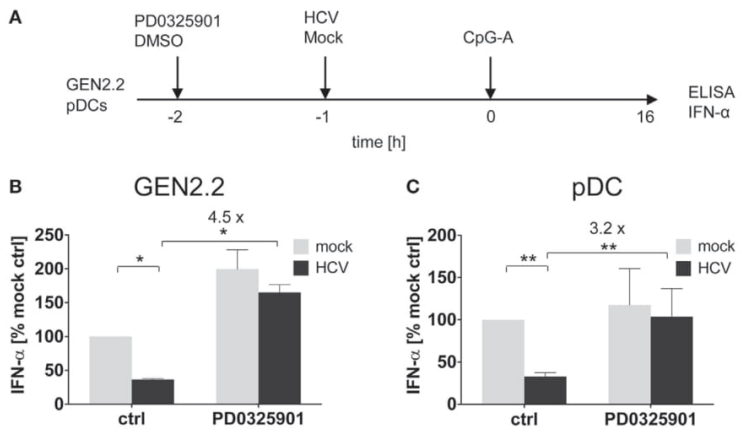

FIGURE 4 | Effect of MEK1/2 inhibition on the hepatitis C virus (HCV) blockade of IFN-α in GEN2.2 cells or primary plasmacytoid dendritic cells (pDCs). **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells **(B)**, or primary pDCs **(C)**, were incubated with 1 μM MEK1/2 inhibitor PD0325901 for 1 h and then treated with HCV virions at MOI = 10 geq/cell for 1 h before CpG-A stimulation. After a 16 h 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 PD0325901. The data show mean ± 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.

MEK1/2 Inhibitor Restores TLR9-Mediated IFN-α Production Blocked by Ligation of RRs with BST2 Expressing HEK293T Cells

ILT7 is another pDC-specific receptor with a regulatory function that signals through the BCR-like pathway and inhibits TLR-mediated IFN-α production (11). In order to evaluate the restoration effect of MEK1/2 inhibitors, we exposed GEN2.2 cells to a HEK293T cell line which expressed BST2, a natural ligand of ILT7 (11), in approximately 95% of cells (**Figure 5A**; Figure S5 in Supplementary Material). In the absence of MEK1/2 inhibitor, the co-culture of GEN2.2 cells with the BST2 expressing HEK293T inhibited IFN-α production induced by CpG-A to 47.4% ($p = 0.001$, **Figure 5B**). When the GEN2.2 cells were exposed to 1 μM PD0325901 prior to co-culture with BST2 expressing HEK293T cells and CpG-A simulation, the IFN-α production significantly increased (4.7-fold, $p = 0.001$, **Figure 5B**). In conclusion, the MEK1/2 inhibitor restored TLR9-mediated IFN-α production blocked by ligation of RR ILT7 with BST2.

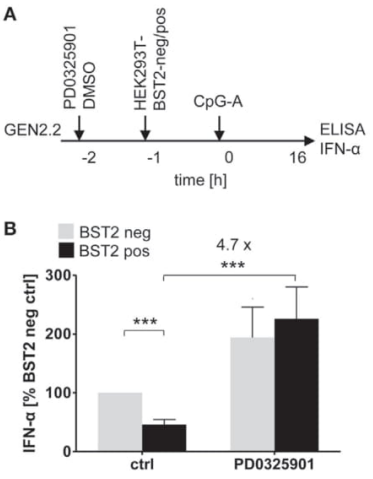

FIGURE 5 | Effect of MEK1/2 inhibition on the blockade of IFN-α by co-culture of GEN2.2 cells with BST2-expressing HEK293T cells. **(A)** Experimental outline. In total 10^5 GEN2.2 cells pretreated with 1 μM PD0325901 were added to a monolayer of 10^5 control HEK293T cells or to the same amount of BST2-expressing HEK293T cells in a volume of 200 μl. The proportion of BST2-expressing cells in the lentivirus-transduced HEK293T cells was determined by flow cytometry using the anti-BST2-PE antibody (Figure S4 in Supplementary Material). The co-cultures of GEN2.2 and HEK293T cells were kept for 1 h at 37°C before adding 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 IFN-α level induced in GEN2.2 cells by CpG-A in co-culture with the mock-transduced BST2-negative HEK293T cells and in the absence of PD0325901. The data show mean ± SEM of five independent co-culture experiments of GEN2.2 cells with BST2-negative or BST2-positive HEK293 cells, *, $p < 0.05$; ***, $p < 0.001$; two-tailed Mann–Whitney test.

MEK1/2 Inhibitor Restores TLR9-Mediated IFN-α Production Blocked by PMA

A recent study showed that treatment of pDCs with PMA, an agonist of PKC activating MEK1/2-ERK signaling pathway, has led to a dose-dependent reduction of IFN-α secretion (34). We investigated the capacity of PD0325901 to reverse the inhibitory effect of PMA on TLR9-mediated IFN-α production (**Figure 6A**). In the absence of the MEK1/2 inhibitor, the production of IFN-α induced in GEN2.2 cells by CpG-A was suppressed by PMA to 25% ($N = 6$, $p = 0.0022$, **Figure 6B**). PD0325901 significantly potentiated CpG-A-induced production of IFN-α in GEN2.2 cells (1.56-fold, $N = 6$, $p = 0.0022$, **Figure 6B**). PD0325901 completely restored the production of IFN-α inhibited in GEN2.2 cells by

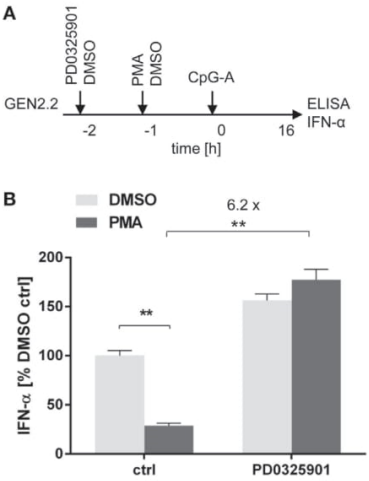

FIGURE 6 | Effect of MEK1/2 inhibitor PD0325901 on the blockade of IFN-α production in phorbol myristoyl acetate (PMA)-stimulated GEN2.2 cells. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated with the MEK1/2 inhibitor for 1 h before stimulation with PMA. After 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 by CpG-A in the presence of DMSO 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.

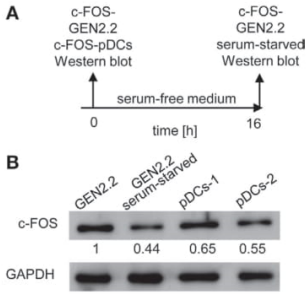

FIGURE 7 | c-FOS in proliferating GEN2.2 cells, GEN2.2 cells starved in the serum-free medium and in primary plasmacytoid dendritic cells (pDCs). **(A)** Experimental outline. Total cell extracts were prepared from GEN2.2 cells immediately after their separation from MS-5 feeder cells (GEN2.2), GEN2.2 cells separated from MS-5 feeder cells and starved overnight in the serum-free medium (GEN2.2 serum-starved), and from primary pDCs isolated from two healthy donors by magnetic-bead purification without any further culture (pDCs-1, pDCs-2). **(B)** c-FOS levels were determined in the total cell extract by Western blotting by rabbit polyclonal Ab c-FOS (sc-52). The values shown below each band represent relative quantity of c-FOS determined by densitometry normalized to proliferating GEN2.2 cells. GAPDH was used as a loading control.

PMA (6.18-fold, $p = 0.0022$, **Figure 6B**). In conclusion, activation of MEK1/2-ERK pathway by PMA inhibited the TLR9-mediated IFN-α production and this effect was abrogated by PD0325901.

c-FOS Levels in pDC Cell Line GEN2.2 Are Higher Than Those in Primary pDCs

The implication of MEK1/2 in the crosstalk of BCR-like and TLR7/9 signaling led us to investigate the role of c-FOS, a downstream immediate early response gene (20), in the regulation of TLR7/9 response. To this end, we compared the levels of c-FOS protein in the GEN2.2 cell line with those in primary pDCs (**Figures 7A,B**). We found that the quantity of c-FOS in GEN2.2 cells cultured in complete medium was approximately double that of primary pDCs (**Figure 7B**). Among numerous transcription factor binding sites in the upstream promoter region of *c-FOS*, the serum response element plays a central regulatory role in responding to external stimuli by growth factors and mitogens (20). To assess the basal level of c-FOS in GEN2.2 cells, we determined c-FOS levels in GEN2.2 starved for 16 h in serum-free medium. The starvation reduced the quantity of c-FOS in GEN2.2 cells to the level present in primary pDCs (**Figure 7B**).

Expression of c-FOS Induced by BDCA-2 Crosslinking Precedes and Exceeds That Induced by CpG-A

We determined the effect of CpG-A and BDCA-2 mAb on the kinetics of expression of the *c-FOS* gene in the serum-starved GEN2.2 cells pretreated or not with PD0325901 (**Figure 8A**). The peak of *c-FOS* transcription occurred 60 min after stimulation with CpG-A (**Figure 8B**), while crosslinking of BDCA-2 induced an earlier (30 min) and a stronger transcription of *c-FOS* (**Figure 8C**). Pretreatment with PD0325901 blocked the induction of *c-FOS* transcription by both CpG-A and BDCA-2 mAb (**Figures 8B,C**). In addition to the quantification of *c-FOS* mRNA by qRT-PCR, we determined the c-FOS protein levels in the serum-starved GEN2.2 cells exposed to CpG-A or BDCA-2 mAb by western blot (**Figures 8D,E**). While stimulation of GEN2.2 cells with CpG-A decreased the level of c-FOS protein (0.82-fold), crosslinking of BDCA-2 increased the production of c-FOS (1.46-fold).

PD0325901 Inhibits G1/S Phase Transition of GEN2.2 Cell Cycle

While pDC line GEN2.2 shares many features with primary pDCs (15, 25–30), GEN2.2 cells principally differ from primary pDCs by their capacity to proliferate. To further analyze this difference, we tested whether the higher basal level of c-FOS in proliferating GEN2.2 cells relative to primary pDCs is related to the MEK1/2-ERK-mediated c-FOS induction and G1/S phase transition of the cell cycle (21) (**Figures 9A,B**). Proliferating GEN2.2 cells were treated with PD0325901, corresponding concentration of DMSO, CpG-A, and BDCA-2 mAb, or starved in serum-free medium, and the impact on their cell cycle was analyzed 16 h later. Cell cycle of a control culture of GEN2.2 cells was analyzed immediately after separation from MS-5 cells. We found that the MEK1/2-ERK pathway inhibitor PD0325901 blocked the cell cycle in proliferating GEN2.2 cells. The cell cycle was also strongly inhibited in the serum-starved GEN2.2 cells, although the impairment of the cell cycle in this cell culture did not permit to calculate residual S phase and G2/M phase cells according to mathematical model used in our analyses. As expected, BDCA-2 crosslinking did not block,

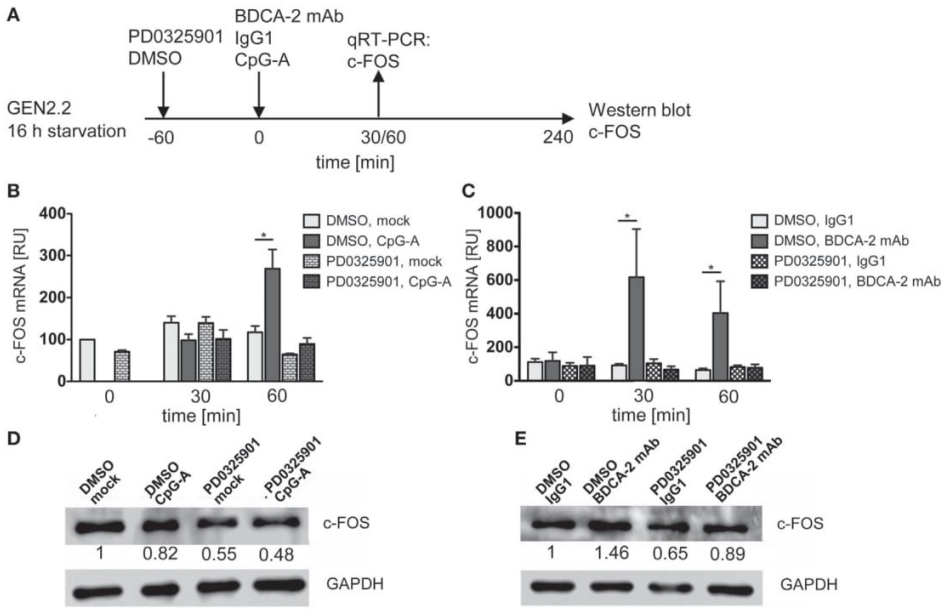

FIGURE 8 | *c-FOS* mRNA and protein expression in GEN2.2 stimulated with TLR9 or RR agonists. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then exposed to CpG-A **(B,D)** or blood dendritic cell antigen 2 (BDCA-2) mAb **(C,E)**. **(B,C)** The expression of human *c-FOS* mRNA was quantified after 30 or 60-min exposure to CpG-A **(B)** or BDCA-2 mAb **(C)** by TaqMan qRT-PCR in the total cellular RNA. The data normalized to time zero show mean ± SEM of three independent experiments; *, $p < 0.05$; two-tailed Mann–Whitney test. **(D,E)** c-FOS protein levels were determined after 240 min exposure to CpG-A **(D)** or BDCA-2 mAb **(E)** in the total cell extract by Western blotting by rabbit polyclonal Ab c-FOS (sc-52). Relative quantity of c-FOS protein normalized to mock-treated GEN2.2 cells determined by densitometry is shown below each band. GAPDH was used as a loading control (representative result of three independent experiments).

but stimulated G1/S phase transition, consistently with increase of c-FOS level in BDCA-2-crosslinked cells (**Figure 8E**). CpG-A stimulation had only slight effect on G1/S phase transition. Cell cycle arrest in the GEN2.2 cells pretreated with PD0325901 or starved for serum (**Figure 9B**) correlated with the decline in the c-FOS level (**Figures 7** and **8D,E**) and with the potentiation of CpG-A-induced production of IFN-α (**Figure 1C**).

BDCA-2 Crosslinking Induces Phosphorylation of c-FOS

It was reported that ERK1/2-mediated post-translational phosphorylation enhances c-FOS stability and transcriptional activity (20, 22, 23). We assessed the phosphorylation of ERK1/2 at T202/Y204 and c-FOS at T325 in serum-starved GEN2.2 cells treated with RR agonist BDCA-2 mAb, TLR9 agonist CpG-A, and PKC agonist PMA (**Figure 10A**). c-FOS phosphorylation was analyzed using Western blotting with the P(T325)-c-FOS antibody. In the control experiment, 15 or 60 min exposure of GEN2.2 cells to PMA-induced strong phosphorylation of ERK1/2 at T202/Y204 and the c-FOS at T325, which was efficiently inhibited by PD0325901 (**Figure 10B**). The levels of total c-FOS and ERK1/2 remained unchanged in GEN2.2 cells stimulated with PMA for 15 or 60 min (Figure S6 in Supplementary Material). Stimulation with BDCA-2 mAb induced strong phosphorylation of ERK1/2 at T202/Y204 and the c-FOS phosphorylation at T325, which was abrogated by pretreatment with MEK1/2 inhibitor PD0325901 (**Figure 10C**; Figure S7 in Supplementary Material). In contrast to BDCA-2 mAb or PMA, CpG-A-induced ERK-1/2 T202/Y204 phosphorylation without inducing the phosphorylation of c-FOS T325 (**Figure 10D**). In conclusion, all three agonists induced phosphorylation of ERK-1/2, which was inhibited by 1 μM PD0325901. BDCA-2 mAb and PMA induced phosphorylation of c-FOS while CpG-A did not. The phosphorylation of c-FOS was inhibited by PD0325901, which is consistent with the regulation of c-FOS by MEK1/2-ERK signaling.

BDCA2 Crosslinking in GEN2.2 Cells and Primary pDCs Induces Upregulation of c-FOS

A recent study reported that BDCA-2 crosslinking and internalization result in up to 16 hr-lasting resistance of pDCs to TLR7/9-mediated stimulation suggesting a stability of the IFN-I inhibitory signal (35). Although c-FOS expression is usually rapid and transient, c-FOS stability is enhanced by phosphorylation (20, 22, 23). These observations led us to investigate the stability of c-FOS levels after stimulation of the BCR-like or TLR9 pathways. We analysed the quantity of c-FOS in the GEN2.2 cell line 16 h after stimulation with the control PMA, BDCA-2 mAb, and CpG-A by flow cytometry in the presence or absence of PD0325901 (**Figure 11A**). The results show that stimulation with PMA and

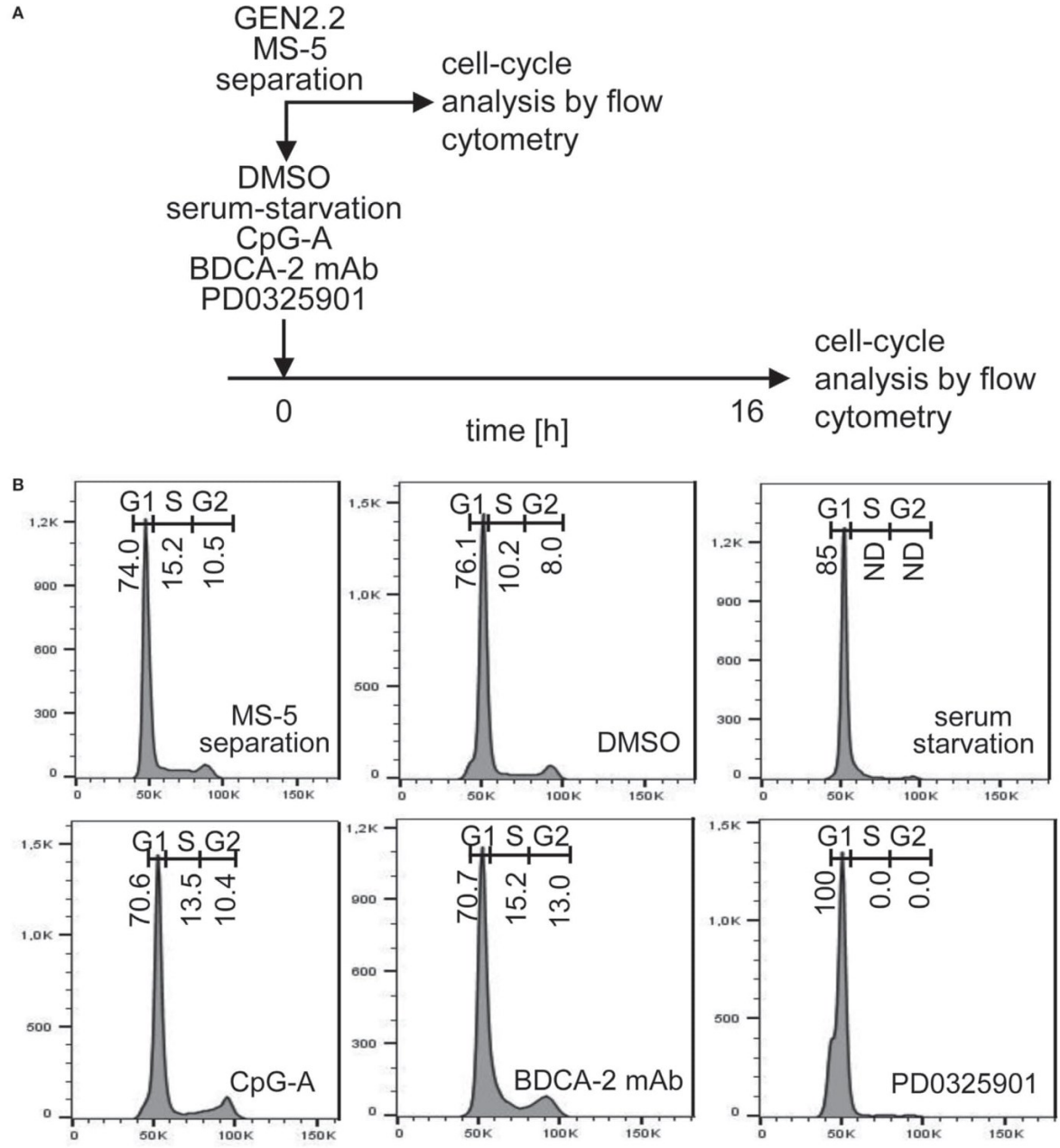

FIGURE 9 | Cell cycle analysis of GEN2.2 cells cultured in the presence of PD0325901, CpG-A, blood dendritic cell antigen 2 (BDCA-2) mAb, or in serum-free medium. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were exposed for 16 hr to 1 μM PD0325901, 4 μg/ml CpG-A, 0.25 μg/ml BDCA-2 mAb, or DMSO (mock-treated control), or analyzed immediately after separation from MS-5 cells. **(B)** Cell cycle analysis using Hoechst 33342 stain. Histograms of live GEN2.2 cells (representative result of three independent experiments) stained with Hoechst 33342 dye showing DNA content distribution. Live/Dead cell discrimination was performed by Zombie Green™ Fixable Viability Kit. ND, not determined.

BDCA-2 mAb induced a sustained increase in c-FOS levels, while stimulation with CpG-A did not (**Figure 11B**). The increase in c-FOS levels in the PMA and BDCA-2 stimulated GEN2.2 cells was inhibited by PD0325901. MFI of c-FOS significantly increased after BDCA-2 crosslinking and PMA stimulation of GEN2.2 cells but not after stimulation with CpG-A ($N = 3$, **Figure 11C**). While

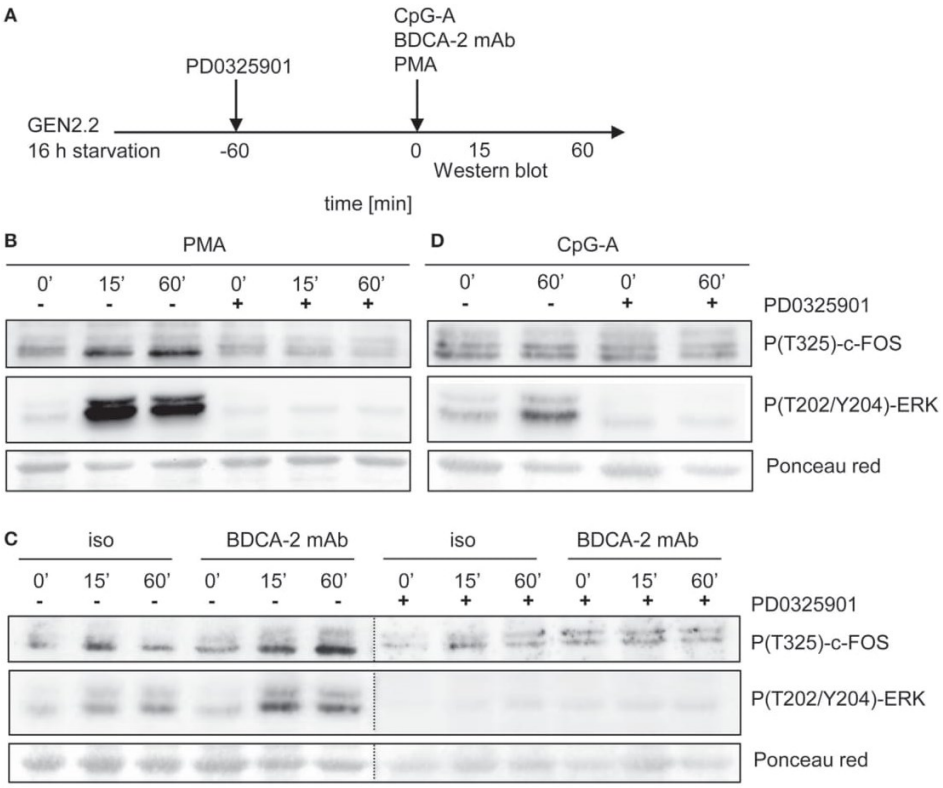

FIGURE 10 | Activation of c-FOS and ERK in GEN2.2 cells stimulated with phorbol myristoyl acetate (PMA), blood dendritic cell antigen 2 (BDCA-2) mAb and CpG-A. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then stimulated with PMA **(B)**, BDCA-2 mAb **(C)**, or CpG-A **(D)**. The activation of c-FOS was evaluated by analysis of c-FOS phosphorylation using Western blotting with the P(T325)-c-FOS antibody. The phosphorylation of ERK-1 was determined by P(T202/Y204) ERK-1. Ponceau red was used as a loading control. Figure **(C)** is composed of two images of two different gels with samples from the same experiment. The two images are separated by a dotted line. Full scans of the original gels are shown in Figure S7 in Supplementary Material.

PD0325901 almost completely inhibited c-FOS production in GEN2.2 cells stimulated by PMA, it exerted only partial inhibition in BDCA-2 mAb-crosslinked cells.

To assess whether stimulation of BDCA-2 in primary pDCs also upregulates the expression of c-FOS, we exposed PBMCs from three healthy donors to BDCA-2 mAb and determined the level of c-FOS in a rapidly dying population of primary pDCs 4 hr later. Because the low proportion of pDCs in PBMCs makes their biochemical analyses difficult, we used flow cytometry for this purpose (**Figures 11A, D–F**). The MFI of c-FOS induced by BDCA-2 mAb increased 2.19 ± 0.85 times compared to isotypic IgG1 control in pDCs (**Figure 11E**). These results show that the stimulation of RRs of pDCs results in a sustained increase of the c-FOS level not only in the GEN2.2 cell line but also in primary pDCs.

DISCUSSION

Our results demonstrate the important role of MEK1/2-ERK signaling in the RR-mediated inhibition of IFN-α and IL-6 production in pDCs. We showed that MEK1/2 inhibitors PD0325901 and U0126 were the only constituents of the panel of inhibitors of BCR-like signaling that not only did not abrogate, but even stimulated TLR9 signaling in GEN2.2 cells. Pharmacological targeting of MEK1/2 in GEN2.2 cells or primary pDCs significantly abrogated inhibition of the TLR9-mediated production of IFN-I induced by BCR-like or PKC signaling. Both BCR-like and PKC signaling activated MEK1/2-ERK pathway.

The molecular mechanism by which the ligation of the RRs antagonizes TLR7/9 signaling in pDCs remains elusive despite years of intense research in many laboratories (8–10, 12–14, 16, 35). We show here that MEK1/2-ERK signaling upregulated the production and phosphorylation of c-FOS. Thus, the potentiation of IFN-I by PD0325901 treatment of GEN2.2 cells could be consequence of a natural role of c-FOS in cell proliferation. The role of c-FOS in the activation of the G1/S cell cycle transition and in the inhibition of IFN-α and IL-6 production in GEN2.2 cells should be further investigated. A higher level of c-FOS in proliferating GEN2.2 cells in comparison with resting primary

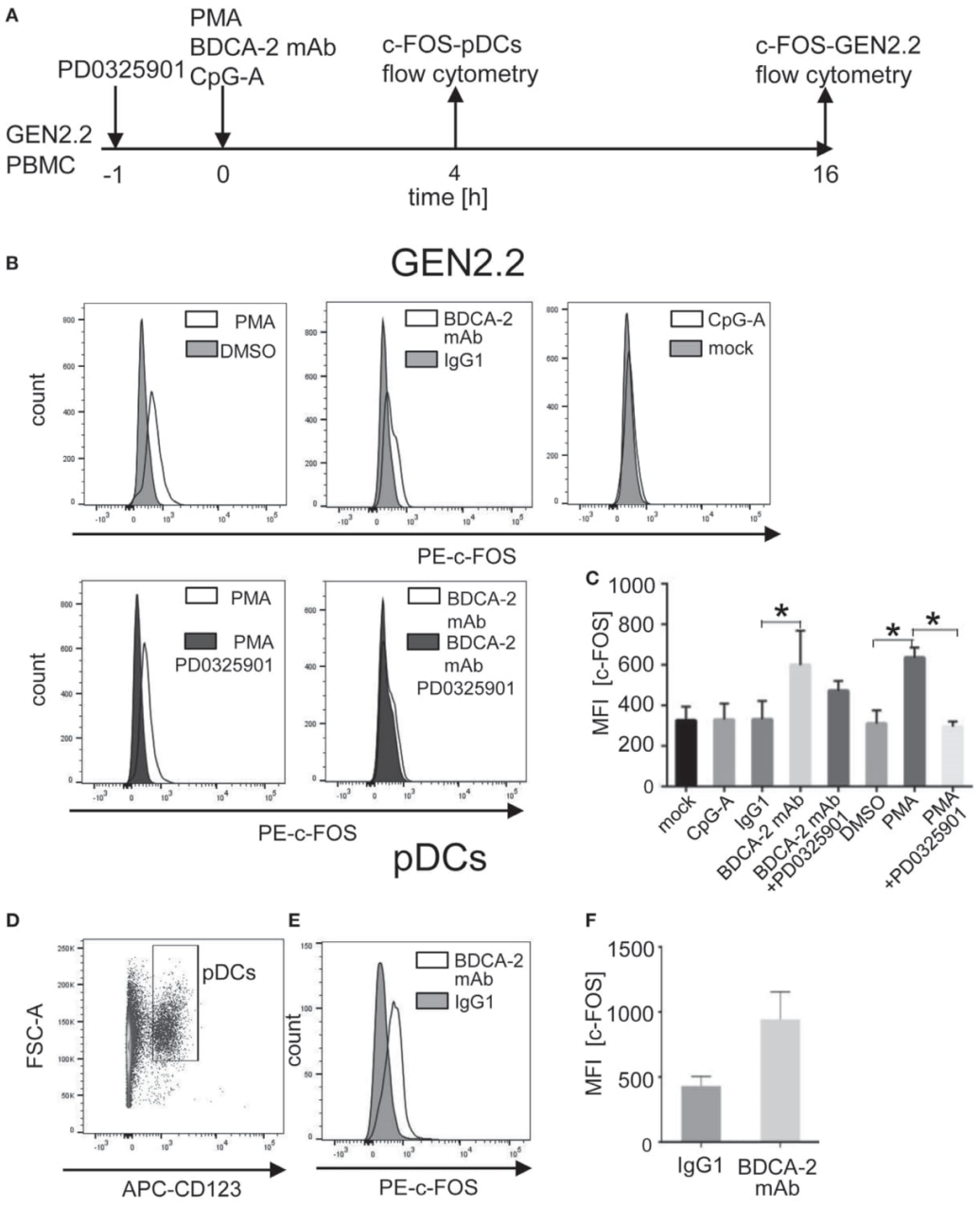

FIGURE 11 | Induction of total c-FOS in GEN2.2 cells and primary plasmacytoid dendritic cells (pDCs). **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells exposed or not to PD0325901 were stimulated with phorbol myristoyl acetate (PMA), blood dendritic cell antigen 2 (BDCA-2) mAb, or CpG-A for 16 h. Peripheral blood mononuclear cells (PBMCs) of healthy donors were exposed to BDCA-2 mAb or IgG1 isotype Ab and quantity of the total c-FOS in pDCs gated from PBMCs was determined 4 h later. **(B)** Fluorescence intensity of the total c-FOS in GEN2.2 cells. Viable GEN2.2 cells were gated according to Live/Dead Zombie Green kit, semipermeabilized and stained with PE-conjugated c-FOS (9F6) rabbit mAb. Control light shaded areas show c-FOS in unstimulated PD0325901 mock-treated GEN2.2 cells. Dark shaded areas show c-FOS in PD0325901-treated GEN2.2 cells. Representative result of three independent experiments. **(C)** The data show mean MFI ± SEM of the total c-FOS in GEN2.2 cells determined in three independent experiments. *, $p < 0.05$; unpaired, two-tailed *t*-test. **(D)** Primary pDCs in PBMCs were gated negatively for Zombie green- (living cells) and FITC-Lin$^-$ and positively for APC-CD123+. **(E)** c-FOS in semipermeabilized primary pDCs gated from PBMCs stimulated with BDCA-2 mAb or IgG1 isotype for 4 h was stained with PE-c-FOS (9F6) rabbit mAb. Representative result of three independent experiments with PBMCs from different healthy donors. **(F)** The data show mean MFI ± SEM of the total c-FOS in primary pDCs gated from BDCA-2-stimulated or unstimulated PBMCs determined in three independent experiments in PBMCs of different donors.

pDCs represents a major difference between these cell types and is consistent with the different outcome of MEK1/2-ERK inhibition. The demonstration of the synergistic effect of MEK1/2 inhibitors on the CpG-A-induced production of IFN-α suggests that under steady-state conditions a natural intrinsic block regulated by MEK1/2 controls the IFN-α level in GEN2.2 cells to a higher level than that in primary pDCs. Release of this block could be a part of the restoration mechanism of IFN-α by MEK1/2 inhibitors in pDCs exposed to RR agonists.

The levels of inhibition of IFN-I production by crosslinking of RR and their restoration by MEK1/2-ERK inhibitors varied depending on the RR ligand. This could be related to differences in the cell-surface distribution of targeted receptors (BDCA-2, ILT7, DCIR) and avidity of tested ligands (BDCA-2 and ILT7 mAbs, HCV particles, or BST2 expressing cells). Among them, BDCA-2 mAb was the most potent inhibitor of IFN-I production. Surprisingly the relative levels of inhibition and restoration of IFN-I production were similar in GEN2.2 cell line and primary pDCs. In addition to differences in receptor/ligand interactions, the levels of inhibition and restoration of IFN-I production were dependent on the mechanism of stimulation of MEK1/2-ERK pathway by BCR-like or PKC signaling. While pretreatment with PD0325901 led to almost complete inhibition of c-FOS expression induced by PMA, c-FOS expression induced by BDCA-2 mAb was only partially inhibited. This suggests that expression of c-FOS induced by BDCA-2 crosslinking and internalization could be partially MEK1/2-ERK independent.

MEK1/2 inhibitor PD0325901 potentiated production of IFN-α in pDC cell line GEN2.2 stimulated by both synthetic (CpG-A and CpG-B) and natural (HSV-1 and HCMV) agonists. In the absence of PD0325901, exposure of pDCs to HSV-1 and HCMV results in a non-permissive infection and TLR9-mediated production of IFN-α (36, 37). Interestingly, the quantity of IFN-α produced by murine pDCs exposed to murine CMV (MCMV) is down-modulated by MCMV-induced stimulation of DAP12, an adaptor molecule of murine RR (38). Recent study demonstrated that EBV and double-stranded DNA viruses induce TRIM29 leading to suppression of IFN-α production (39). The potential role of TRIM29 in HSV-1 and HCVM-mediated inhibition of IFN-α production in pDCs needs to be clarified.

A previous report implicated c-FOS induced by MAP3-kinase TPL-2 in the negative regulation of TLR9-mediated production of IFN-β in mouse macrophages and myeloid (mDCs), but not in mouse pDCs (24). In contrast, we show here that c-FOS induced by MEK1/2-ERK signaling is involved in the regulation of TLR9 signaling in human pDCs. It is possible that TPL-2 and MEK1/2-ERK signaling are interpreted differently in mouse and human pDCs compared with macrophages and mDCs as a consequence of an interaction of ERK activation with other signaling pathways triggered by TLR9 (18). Several cell type-specific studies have shown that the interaction of TLR7/9 with BCR-like signaling may be regulated in a different way in human pDCs (7, 12, 14, 16, 35, 40).

Activation of Ras/MEK1/2/ERK downregulates expression of IFN-I also in human epithelial cancer cells (41). Together with our experiments, these results suggest that MEK1/2-ERK signaling can play a general role in regulation of IFN-I. Another recent study demonstrated that MEK1/2-ERK-mediated phosphorylation of c-FOS in HCV-infected hepatocytes induced miR-21, which targeted MyD88 and IRAK1 and contributed to the suppression of IFN-I production (42). We did not detect a significant increase of miR-21 level in GEN2.2 cells exposed to BDCA-2 mAb or CpG-A (not shown).

We have demonstrated that inhibitors of MEK1/2 restore the production of IFN-I inhibited by ligation of RRs with HCV particles or with BST2 expressing cancer cells. These results suggest that pharmacological targeting of MEK1/2-ERK signaling could be a strategy to overcome immunotolerance of pDCs and re-establish their immunogenic activity. This finding complements our previous results showing that an inhibitor of SYK, a protein kinase involved in both TLR7/9 and BCR-like pathways, could be a useful tool to suppress the overproduction of IFN-I and to re-establish tolerogenic homeostatic functions of pDCs (15). The role of IFN-I in the pathogenesis of chronic viral infections and cancer is unclear and ambivalent. IFN-I responses are critical in the early phases of immune response to infections, but the chronic and systemic activation of pDCs can paradoxically lead to deleterious consequences for the immune system (43, 44). It is likely that an intense signaling occurs in the mucosa, involving a local accumulation of pDCs producing IFN-I early during HIV-1 infection, which is associated with the chronic activation of the immune system (45, 46). While in this era of great success of direct-acting antivirals against HIV and HCV the stimulation of IFN response might represent an adjuvant therapy, important namely in the case of virus escape, the induction of IFN-I in combination with existing antivirals may cure HBV infection (47–49). IFN-I also plays an important role in antitumor immunity (3, 50). The addition of exogenous IFN-α reverts the immunotolerance of tumor-associated pDCs in breast and ovarian carcinoma (4, 51). Pharmacological targeting of MEK1/2 signaling may constitute an attractive new approach to study mechanisms of modulation of pDC activation in pathophysiological conditions such as chronic viral infections and cancer.

MATERIALS AND METHODS

Isolation and Culture of Primary pDCs

Peripheral blood mononuclear cells (PBMCs) from healthy anonymous donors were obtained from the national blood services (Etablissement Francais du Sang, Marseille, France). Blood samples were obtained after written consent following the approval of the EFS, Marseille, France, and the Center de Recherche en Cancérologie de Marseille (CRCM) in accordance to the convention signed the 20th May 2014. pDCs purified from PBMCs as described previously were 75–95% pure, with a contamination of less than 5% mDCs (32, 33, 52, 53). Isolated pDCs were cultured in RPMI 1640 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.

pDC Line GEN2.2

Human pDC line GEN2.2 (25) 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, the dynamic flow cytometry and the Western blot experiments, GEN2.2 cells were separated from the MS-5 feeder cells.

Inhibitors, Antibodies, and Reagents

MEK-1/2 inhibitor PD0325901 obtained from InvivoGen (Toulouse, France) and U0126 obtained from Sigma (Sigma-Aldrich, Lyon, France) were used as recommended by supplier. PD0325901 is a selective non-ATP-competitive allosteric MEK1/2 inhibitor with *in vitro* IC50 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* IC50 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 (all InvivoGen, San Diego, USA), and BDCA-2 antibody (Miltenyi Biotech, Paris, France), and ILT7 antibody (eBioscience) were used.

In Vitro pDC 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 stimulated with 4 μg/ml CpG-A or CpG-B, 25 ng/ml PMA, 20 μg/ml of BDCA-2 or ILT7 antibody, or 10 HCV geq/cell for 16 h. In some experiments, BDCA-2 or ILT7 antibody-exposed cells were further crosslinked with goat-antimouse $F(ab')_2$ (15 μg/ml) (Jackson ImmunoResearch).

Production and Purification of Cell Culture-Derived HCVcc (JFH-1 3 M), HSV-1, and HCMV Virus Stocks

Hepatitis C virus cc particles were prepared in Huh7.5 cells (55) (kindly provided by APATH L.L.C.) on the basis of plasmid pJFH-1 displaying mutations, F172C and P173S in core and N534K in E2 (56), as described previously (33). The ultracentrifuged virus purified through a cushion of 20% sucrose was resuspended in RPMI 1640 to obtain a 1,000-fold concentrated virus suspension containing 10^7 $FFU_{Huh7.5}/10^{11}$ HCV RNA copies/ml. Stocks of HSV-1, strain Praha, and HCMV, strain AD-169, were prepared as described previously (57, 58).

Preparation of BST2 Expressing HEK293T Cells

The BST2 sequence from pCMV-Sport6-BST2 was cloned into the pRRL.PPT.SF.i2GFPp expression vector to produce a lentiviral vector pRRL-BST2-GFP. HEK293T cells were transduced by the resulting lentivirus construct at MOI = 10 and GFP-positive cells were selected by FACSAria (BD Biosciences). The expression of GFP and BST2 in transduced cells was determined by flow cytometry by LSRII (BD Biosciences).

Determination of *c-FOS* Expression

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Human *c-FOS* was amplified with SYBR® Green PCR Master Mix (Applied Biosystems) using the following primers: *c-FOS*: forward: 5′-CAAGCGGAGACAGACCAACT-3′and reverse 5′-AGTCAGATCAAGGGAAGCCA-3′; GAPDH: forward: 5′-GCGAGATCCCTCCAAAATCAA-3′and reverse 5′-GTTCACACCCATGACGAACAT-3′. Relative expression levels were calculated using $2^{-\Delta\Delta CT}$ method. *GAPDH* was used as endogenous control.

Determination of ERK and c-FOS by Immunobloting

Total c-FOS and ERK in the whole cell lysate of GEN2.2 cells or primary pDCs were determined by Western blotting by means of rabbit polyclonal c-FOS (sc-52) and ERK1/2 (sc-154) Abs (Santa Cruz Biotechnology, Dallas, USA). Phosphorylation of ERK and c-FOS in the whole cell lysate of GEN2.2 cells was analyzed by Western blotting using phospho-c-FOS-T325 Ab from Abcam (Cambridge, UK) and ERK Ab T202/Y204 (Santa Cruz Biotechnology, Dallas, USA) as described previously (15). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the membranes were washed and the protein bands were detected with Super Signal™ enhanced chemoluminiscent substrate detection reagent (ThermoFisher Scientific, Villebon-sur-Yvette, France). Densitometric analyses were performed using Amersham Imager 600 (GE Healthcare Life Science). Band intensities were normalized to GAPDH or Ponceau red.

Determination of c-FOS by Dynamic Flow Cytometry

To determine total c-FOS by dynamic flow cytometry, 10^6 GEN2.2 cells or 2×10^6 PBMCs per milliliter were kept in the RPMI 1640 medium supplemented with 10% FCS. Aliquots of 10^6 GEN2.2 cells or 8×10^6 PBMCs were stimulated with 4 μg/ml CpG-A, 100 ng/ml PMA, 10 μg/ml of BDCA-2 mAb for 16 hr (GEN2.2 cells) or 4 hr PBMCs. Live/Dead cell discrimination was performed by Zombie Green™ Fixable Viability Kit (BioLegend, San Diego, USA). For flow cytometry analysis of total c-FOS, cells were fixed in 4% formaldehyde for 10 min, permeabilized by 90% methanol for 30 min, and stained by PE conjugated c-FOS (9F6) rabbit mAb (Cell Signaling, Danvers, USA). For determination of c-FOS in primary pDCs, PBMCs were stained by APC-conjugated anti-human CD123 mouse mAb (BD Biosciences, San Jose, USA) and FITC-conjugated anti-human lineage cocktail mouse Abs (BioLegend, San Diego, USA). pDCs in PBMCs population were defined as Lin-, CD123+ cell population. Samples were analyzed using a BD LSR FORTESSA cytometer (BD Biosciences, San Jose, USA) and data were processed using FLOWJO software (Treestar, San Carlos, USA).

Cell Cycle Analysis

For analysis of cell cycle, 10^6 GEN2.2 cells/ml of RPMI 1640 medium supplemented with 10% FCS were aliquoted in 1 ml

quantities in 6-well flat-bottom culture plates and exposed to 1 μM PD0325901, 4 μg/ml CpG-A, and 10 μg/ml of BDCA-2 mAb for 16 h. The cells were then resuspended in the RPMI 1640 medium containing 6 μg/ml Hoechst 33342 Dye (ThermoFischer Scientific) and incubated at 37°C in 5% CO_2 for 30 min and the amount of DNA was determined by flow cytometry. Live/Dead cell discrimination was performed by Zombie Green™ Fixable Viability Kit (BioLegend, San Diego, USA). Samples were analyzed using a BD LSR FORTESSA cytometer (BD Biosciences, San Jose, USA) and data were processed using FLOWJO software (Treestar, San Carlos, USA). Phases of the cell cycle were calculated by Dean-Jett-Fox model.

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.

Statistical Analysis

Quantitative variables are expressed as the mean ± SEM (standard error of the mean). To compare the levels of cytokine production and transcription of c-FOS mRNA by pDCs, we used a Mann–Whitney or a Wilcoxon two-tailed non-parametric tests. For flow cytometry analyses, we used two-tailed *t*-test. Data were analyzed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA). A p value ≤ 0.05 was considered to be significant.

ETHICS STATEMENT

Peripheral blood mononuclear cells (PBMCs) from healthy anonymous donors were obtained from the national blood services (Etablissement Francais du Sang, Marseille, France). 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.

AUTHOR CONTRIBUTIONS

Contribution: VJ, BA, and AF-H equally performed research, designed research, and analyzed data. TH, KT, and JW performed research and analyzed data. JN, DO, and PD designed research and analyzed data. LC and JP provided essential materials. RS and IH designed research, analyzed data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00364/full#supplementary-material.

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

Supplementary Material

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

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Supplementary Figures

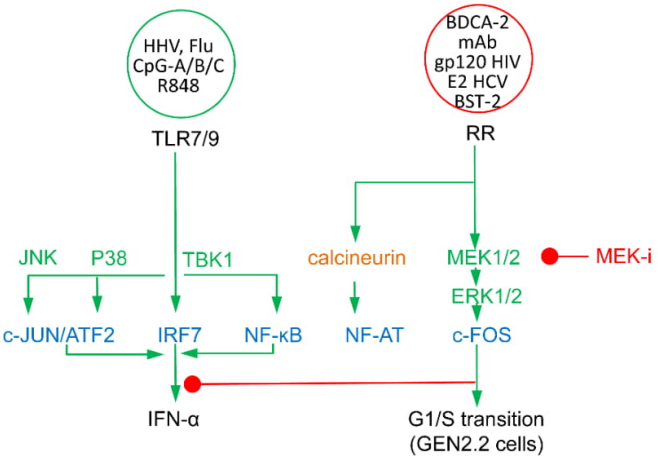

FIGURE S1 | TLR7/9 and BCR-like (RR-triggered) signaling pathways in pDCs. Protein kinases JNK, p38 MAPK, TBK1, MEK1/2 (in green), phosphatase calcineurin (in ochre), and transcription factor NF-κB (in blue) studied in this work are depicted. Transcription factors involved in regulation of expression 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.

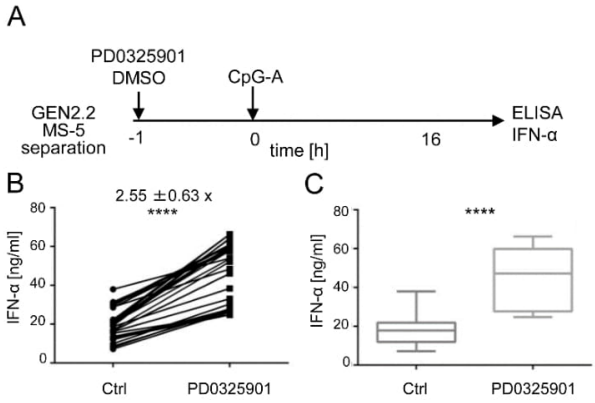

FIGURE S2 | Effect of MEK1/2 inhibitor PD0325901 on the production of IFN-α in CpG-A-stimulated GEN2.2 cells. (A) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated or not with 1 µM PD0325901 for 1 h before stimulation with CpG-A. After a 16 h culture, the IFN-α production 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.

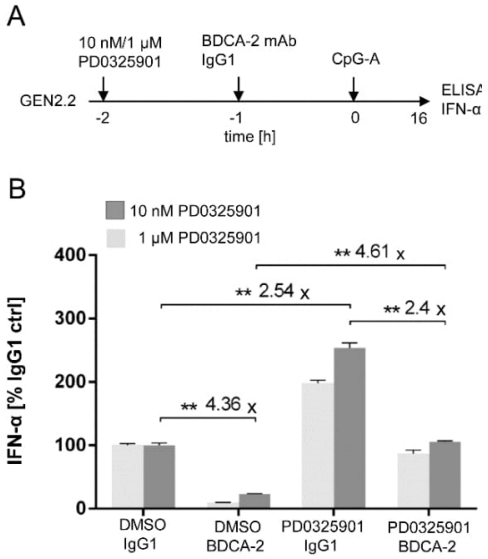

FIGURE S3 | Effect of MEK1/2 inhibitor PD0325901 on the blockade of IFN-α production by ligation of RRs of GEN2.2 cells with BDCA-2 mAb. (A) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated with 10nM or 1 µM PD0325901 for 1 h before stimulation with BDCA-2 mAb and CpG-A. 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.

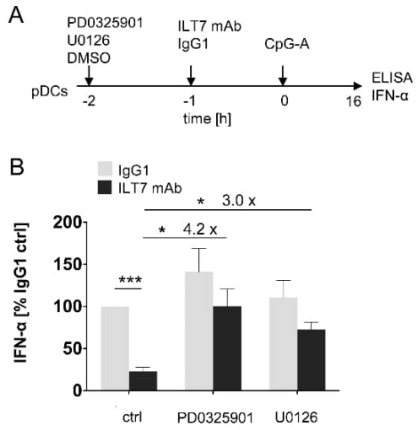

FIGURE S4 | Effect of MEK1/2 inhibitors PD0325901 and U0126 on the blockade of IFN-α production in primary pDCs by ligation of RR ILT7. (A) Experimental outline. Primary pDCs isolated from PBMCs of healthy donors were incubated with 1 µM U0126 (N=5) or 1 µM PD0325901 (N=6) for 1 h before stimulation with ILT7 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.

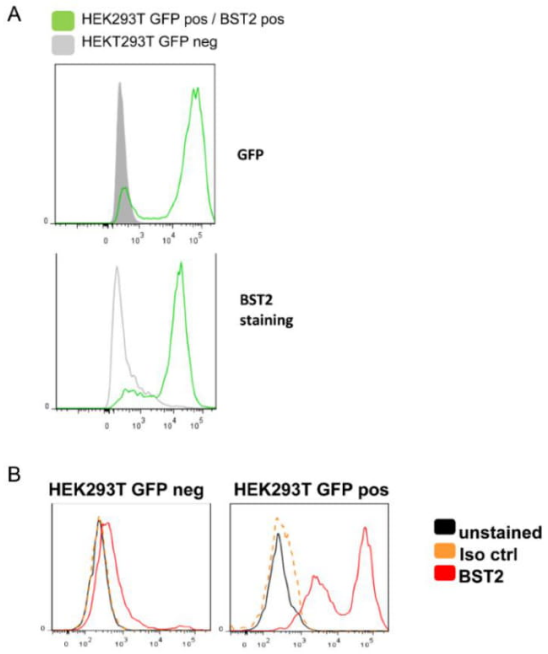

FIGURE S5 | Characteristics of BST2 expressing HEK293T cells. HEK293T cells transduced by lentivirus vector pRRL-BST2-GFP and sorted by GFP marker were analyzed for the expression of GFP and BST2 just after cell sorting **(A)** or after a 2-week culture **(B)**. Both the BST2-transduced (GFP+) cells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

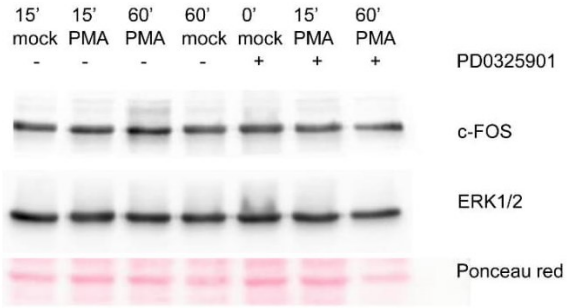

FIGURE S6 | c-FOS and ERK1/2 in PMA-stimulated GEN2.2 cells. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then stimulated with PMA for 0, 15 and 60 min. c-FOS and ERK1/2 were analyzed using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

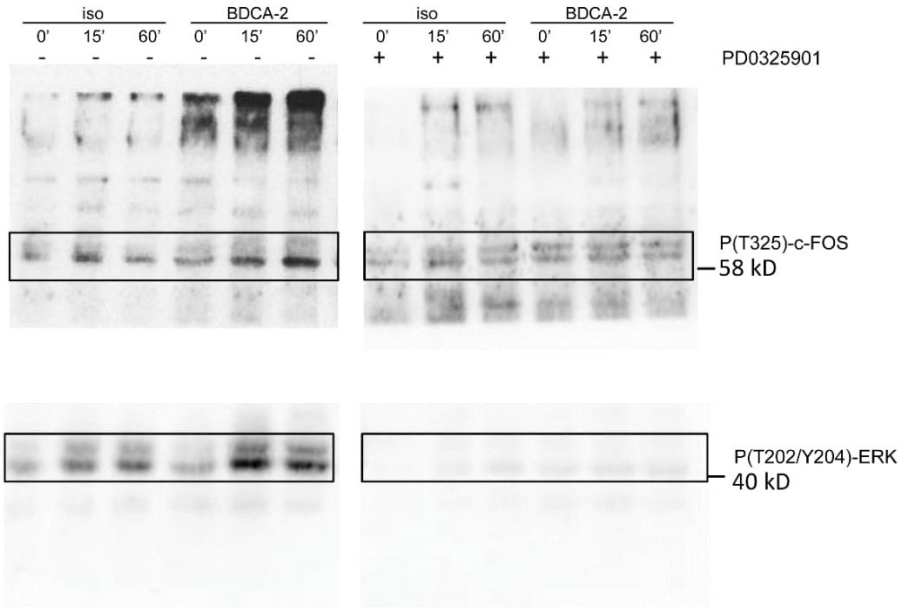

FIGURE S7 | Activation of c-FOS (A) and ERK (B) in GEN2.2 cells stimulated with BDCA-2 mAb – full scans of original gels. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then stimulated with BDCA-2 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**.

9.3 Příloha č. 3

viruses

MDPI

Article

Expression of TIM-3 on Plasmacytoid Dendritic Cells as a Predictive Biomarker of Decline in HIV-1 RNA Level during ART

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Abstract: Depletion and functional impairment of circulating plasmacytoid dendritic cells (pDCs) are characteristic attributes of HIV-1-infection. The mechanism of dysfunction of pDCs is unclear. Here, we studied the development of phenotype of pDCs in a cohort of HIV-1-infected individuals monitored before the initiation and during a 9-month follow up with antiretroviral therapy (ART). Using polychromatic flow cytometry, we detected significantly higher pDC-surface expression of the HIV-1 receptor CD4, regulatory receptor BDCA-2, Fcγ receptor CD32, pDC dysfunction marker TIM-3, and the marker of killer pDC, TRAIL, in treatment-naïve HIV-1-infected individuals before initiation of ART when compared to healthy donors. After 9 months of ART, all of these markers approached but did not reach the expression levels observed in healthy donors. We found that the rate of decline in HIV-1 RNA level over the first 3 months of ART negatively correlated with the expression of TIM-3 on pDCs. We conclude that immunogenic phenotype of pDCs is not significantly restored after sustained suppression of HIV-1 RNA level in ART-treated patients and that the level of the TIM-3 expressed on pDCs in treatment naïve patients could be a predictive marker of the rate of decline in the HIV-1 RNA level during ART.

Keywords: HIV-1; antiretroviral therapy (ART); innate and adaptive immune responses; plasmacytoid dendritic cells (pDCs); pDC dysfunction; T cell Ig and mucin-domain containing molecule 3 (TIM-3); BDCA-2; Toll-like receptors 7 and 9 (TLR7/9)

1. Introduction

Plasmacytoid dendritic cells (pDCs) are a highly-specialized subset of dendritic cells that play a central role at the interface of innate and adaptive immunity. They sense HIV-1 primarily via endosomal Toll-like receptors 7 (TLR7), which recognizes ssRNA [1,2]. TLR signaling leads to the

secretion of proinflammatory cytokines and chemokines such as interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), IL-6, IL-8, and, most importantly, type I IFNs (IFN-I, α/β/ω) [3–6]. In addition to TLR7/9, pDCs express multiple specific receptors that facilitate antigen capture and presentation and regulate pDC function, namely IFN-I production, thus preventing abnormal immune response.

The role of IFN-I and pDCs in the pathogenesis of HIV-1 infection is unclear and ambivalent. IFN-I production is critical in the early phases of the immune response to infections, but the chronic and systemic activation of pDCs can paradoxically lead to deleterious consequences for the immune system [7,8]. It is likely that an intense chronic immune activation occurs in the mucosa, involving the accumulation of pDCs producing IFN-I during HIV-1 infection [9,10]. pDCs suppress HIV-1 replication but contribute to HIV-1 induced immunopathogenesis in humanized mice [11]. It has been shown that HIV-1 infection impairs B and T lymphocyte attenuator (BTLA)-mediated signaling in $CD4^+$ and $CD8^+$ cells dependent on pDC-derived IFN-α, which contributes to broad T-cell hyperactivation [12]. Chronic HIV-1 infection also depletes group 3 innate lymphoid cells (ILC3s) through pDC activation, induction of IFN-I, and CD95-mediated apoptosis, resulting in an increase in bacterial infection and inflammation [13].

In HIV-1 infection, the number of pDCs as well as their function is decreased simultaneously with $CD4^+$ T cell population [14,15]. In contrast to immune activation, pDC dysregulation is directly related to the number of tolerogenic or apoptosis-inducing functions. Thus, upon infection, HIV-1 virions stimulate conversion of pDCs into TNF-related apoptosis-inducing ligand (TRAIL)-expressing IFN-producing killer pDC (IKpDC), which in turn facilitate the apoptosis of $CD4^+$ T cells [16]. pDCs in untreated HIV-1-infected individuals compared to controls and ART-treated patients were also found to express an increased level of programme death ligands (PD-L) 1 and PD-L2 [17]. Another marker of pDC dysfunction in HIV-1-infected individuals, the T cell inhibitory receptor TIM-3 (T cell Ig and mucin-domain containing molecule) has been shown to be associated with the recruitment of IRF7 and p85 to lysosomes and the submembrane displacement of TLR9 [18].

To better understand the mechanisms underlying HIV-1 immunopathogenesis, we analyzed stimulatory, inhibitory, tolerogenic, or apoptosis-inducing functions that are mediated by pDCs in a cohort of HIV-1-infected individuals before and during a 9-month follow-up course of ART. In this cohort, we studied the expression of two HIV-1 receptors, CD4, a key determinant of divergent HIV-1 sensing by pDCs [19] and blood dendritic cell antigen 2 (BDCA-2, CD303, CLEC4C), a lectin-like regulatory receptor [20] which binds to and can be activated by the envelope glycoprotein gp120 of HIV-1 [21]. We also determined the expression of the pDC activation marker HLA-DR, Fcγ receptor CD32, pDC dysfunction marker TIM-3, and the killer pDC marker, TRAIL. We found that the frequency and the mean fluorescence intensity (MFI) of $TIM\text{-}3^+$ pDCs determined before initiation or after 3 months of ART negatively correlated with the rate of decline in HIV-1 RNA level over the 3-month ART. Our data showed that the immunogenic phenotype of pDCs was not significantly restored after sustained suppression of HIV-1 RNA in 9-month ART-treated patients.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Each patient 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).

2.2. Patients

Twenty-one viremic individuals with plasma viremia $\geq 10^4$ RNA copies per milliliter of plasma were enrolled for a period of 9 months at the HIV Clinic of Hospital Na Bulovce (Table 1) 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 1. We had access to the clinical data of these patients including analyses of their lymphocyte populations for another 14 months. Enrollment criteria: HIV-1 infection, $\geq 10^4$ 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).

Table 1. Clinical characteristics of the HIV patient cohort.

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.

2.3. Patients PBMCs

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.

2.4. In Vitro pDC Stimulation

To determine cytokine production, PBMCs aliquoted in 100-µL quantities (10^7 cells/mL) into 96-well round-bottom culture plates and stimulated with 20 µg/mL of BDCA-2 mAb or IgG1 isotype for 2 h and then with 4 µg/mL of CpG-A for 16 h.

2.5. Flow Cytometry Analysis

To carry out the flow cytometric analysis of pDC phenotype, we created a muticolor 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 were 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).

2.6. Statistical Analysis

Quantitative variables were expressed as the mean ± SEM (standard error of the mean). To compare the levels of cytokine production by pDCs, we used either Mann–Whitney or a Wilcoxon two-tailed non-parametric tests. The data was analyzed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). A p value of ≤ 0.05 was considered to be significant.

3. Results

3.1. Persistent Dysfunction of pDCs from ART-Treated HIV-1-Infected Individuals after Sustained Suppression of HIV-1 RNA

Here, we analyzed the main types of immune cells in peripheral blood in a cohort of 21 ART-treated HIV-1-infected individuals (Table 1). We assessed the dynamics of the lymphocyte populations for a period of 23 months (Figure 1). After 3 months of ART, we observed a decrease in plasma HIV-1 RNA level from (median (interquartile range (IQR))) 4.70, IQR (4.57–5.02) $\log_{10}$ copies/mL 1.64, IQR (0.7–1.96) $\log_{10}$ copies/mL, below the level of 2.93 $\log_{10}$ copies/mL of plasma, and it continued to decrease over the remaining 6 months (Figure 1A). The major lymphocyte populations, $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells in healthy donors, treatment naïve HIV-1-infected patients, and ART-treated HIV-1-infected individuals were quantified by flow cytometry (Figure 1B–D). Inversely to HIV-1 RNA level, $CD4^+$ T cell count in blood increased from the median value of 469, IQR (375–531) $CD4^+$ T cells/mm^3 of blood to 748, IQR (609–945) $CD4^+$ T cells/mm^3 of blood (Figure 1E). Similarly, the median value of B cells in blood increased from 159, IQR (137–224) B cells/mm^3 of blood to 214, IQR (136–367) B cells/mm^3 of blood (Figure 1F). Also, the median value of NK cells increased from 230, IQR (169–459) NK cells/mm^3 of blood to 412, IQR (308–541) NK cells/mm^3 of blood (Figure 1G). In contrast to $CD4^+$ T cells, B cells, and NK cells, the median value of $CD8^+$ T cells decreased from 1551, IQR (1070–1737) CD8 T cells/mm^3 of blood to 1005, IQR (713–1555) CD8 T cells/mm^3 of blood (Figure 1H). After 9 months of ART, the median values of $CD8^+$ T cells remained significantly higher than those in healthy donors (HD), while the median values of $CD4^+$ T cells remained significantly lower. The $CD8^+$ T cell levels were significantly increased in comparison to healthy donors even after 23 months of ART.

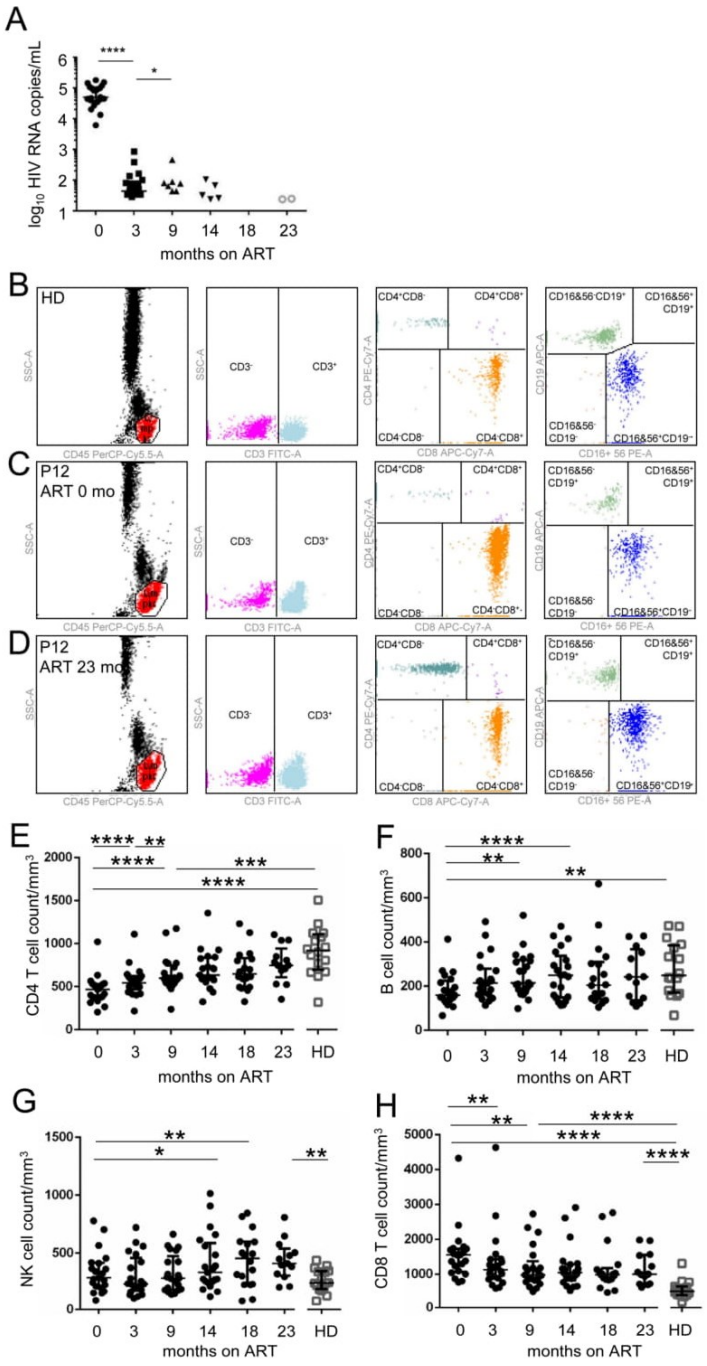

Figure 1. Adaptive immunity is only partially restored over the course of ART despite the sustained suppression of HIV-1 RNA level. Characteristics of the cohort of 21 HIV-infected subjects. (**A**) HIV RNA copies/mL of plasma before and during anti-retrovirus therapy (ART). (**B**–**D**) Dot plots for the quantification of the major lymphocyte populations in peripheral blood of a healthy donor (HD) (**B**), treatment-naïve patient no. 12 (P12) (**C**), the same patient after 23 months of ART (**D**). (**E**) $CD3^+CD4^+$ T cell counts during ART. (**F**) $CD19^+$ B cell counts during ART. (**G**) $CD3^-CD16^+CD56^+$ NK cell counts during ART. (**H**) $CD3^+CD8^+$ 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.

While the dysfunction of $CD4^+$, $CD8^+$ T cells, B cells, and NK cells during the course of HIV infection has been extensively studied, little is known regarding the impairment of dendritic cell function. According to design of this study, we followed the development of pDC cell count and phenotype during 9 months of ART. Previous reports showed that the quantity of blood pDCs is severely reduced in AIDS patients [14,15]. To quantify pDCs, PBMCs were gated according to their size and then into singlets, and after exclusion of dead cells, pDCs were defined as live $Lin^-CD4^+BDCA2^+$ cells (Figure 1A,B). The pDC median number in the cohort of 21 treatment-naïve HIV-1-infected individuals before initiation of ART was 4.08, IQR (2.59–4.90) pDC/mm^3. It was reduced by 54% in comparison to 13 healthy donors (Figure 2B). Nine months of ART partially restored pDC cell count but its median value 5.35, IQR (3.45–7.99) pDC/mm^3 remained significantly lower than that detected in healthy donors (71%, $p = 0.04$). Increase of the median cell number in pDCs (1.31 times, $p = 0.04$, Figure 2A) observed over 9 months of ART was like that observed for $CD4^+$ T cells (1.28 times, $p = 0.008$, Figure 1B).

Then, we investigated the expression of surface markers TIM-3, TRAIL, BDCA-2, HLA-DR, CD32, and CD4 in $Lin^-CD4^+BDCA\text{-}2^+$ pDCs (Figure 2A,C–K). FMO gating was used to quantify the frequency of $TIM\text{-}3^+$ and $TRAIL^+$ pDCs, while the mean fluorescence intensities (MFI) were used to quantify expression of constitutive pDC markers, BDCA-2, HLA-DR, CD32, and CD4. As reported previously, HIV-1 infection in treatment-naïve individuals is associated with elevated frequency of $TIM\text{-}3^+$ pDCs [18]. We found that the frequency of $TIM\text{-}3^+$ pDCs in treatment-naïve individuals exceeded 1.42 times the frequency of $TIM\text{-}3^+$ pDCs in healthy donors ($p = 0.0026$, Figure 2E). The median value of the frequency of $TIM\text{-}3^+$ pDCs showed decreasing tendency over the 9-month ART but their median value remained significantly elevated in comparison to healthy donors (1.2 times, $p = 0.0155$). Similarly, HIV-1 infection in treatment-naïve individuals was associated with elevated median value of the frequency of $TRAIL^+$ pDCs (3.32 times, $p < 0.0001$), which showed decreasing tendency over the 9-month ART (Figure 2F). The frequency of $TRAIL^+$ pDCs in ART-treated patients remained significantly elevated in comparison to healthy donors (2.76 times, $p < 0.0001$).

Then, we analyzed expression of the pDC markers that are constitutively present in pDCs. As shown for BDCA-2, we compared the MFI values of these markers in healthy donors with those of ART-treated HIV-1-infected individuals (Figure 2G). In comparison to healthy donors, HIV-1 infection in treatment-naïve individuals was associated with elevated MFI of BDCA-2 (1.8 times, $p = 0.015$) (Figure 2H), CD32 (1.5 times, $p = 0.046$) (Figure 2J) and CD4 (1.6 times, $p = 0.0013$) (Figure 2K). Among all these variables, only the median values of MFIs of HLA-DR did not significantly vary between healthy donors, treatment-naïve, and ART-treated HIV-1-infected individuals (Figure 2I). The median values of MFIs of these markers showed a decreasing tendency in the course of ART, however, with the exception of CD32, which decreased 1.38 times ($p = 0.044$), they did not reach statistical significance; median values of CD4 and BDCA-2 of ART-treated patients remained significantly elevated in comparison to healthy donors.

Collectively, based on this multifactorial analysis we concluded that after sustained suppression of the HIV RNA level in 9-month ART-treated patients, the median number of pDCs significantly increased, however, their immunogenic phenotype was not significantly restored.

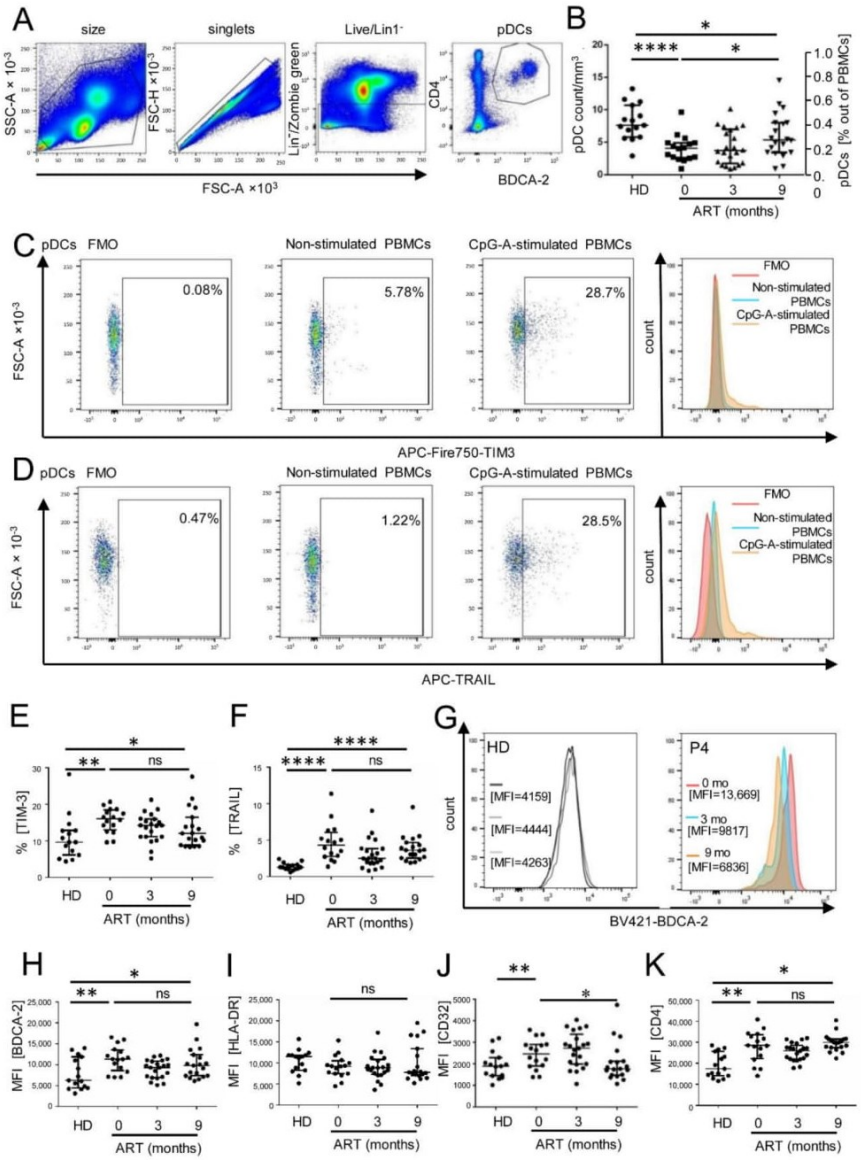

Figure 2. Impaired pDC phenotype persists after sustained suppression of HIV RNA in plasma of ART-treated patients. (**A**) Gating strategy for identification of pDC phenotype: PBMCs were gated according to their size and then into singlets, and after exclusion of dead cells (Zombie green) and Lin1$^+$ 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 TIM-3$^+$ (**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 TIM-3 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 ranges. N = 21. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ns, non-significant; two-tailed Mann-Whitney test.

3.2. Decline in HIV-1 RNA Level after Initiation of ART Correlates with Expression of TIM-3 on pDCs

We defined the rate of decline in HIV-1 RNA level over the 3-month ART as a new additional parameter to evaluate the success rate of ART in HIV-1-infected individuals. To this end we calculated the ratio of plasma HIV-1 RNA copies/mL (virus load, VL) determined in the treatment-naïve individuals at the time zero of ART ($VL_{0\text{-mo}}$) to the plasma HIV-1 RNA copies/mL over the 3-month ART ($VL_{3\text{-mo}}$) (Figure 3A, Table 1). While the HIV-1 RNA level in different patients decreased over the first 3 months of ART by 2.2–4.6 $\log_{10}$ (Figure 3A), $CD4^+$ T cell count increased over the same period of time from 1.1 to 2.8 times (Figure 3B). The rate of decline in HIV-1 RNA level over 3 months did not correlate with the initial virus load in the same individuals (Figure 3C), or with the rate of restoration of $CD4^+$ T cells. We used the rate of decline in plasma HIV-1 RNA copies/mL over 3 months $[VL_{0\text{-mo}}/VL_{3\text{-mo}}]$ $\log_{10}$ as a parameter to characterize individuals that respond more or less rapidly to ART. We subsequently analyzed the distribution of phenotypic markers of pDCs in these HIV-1-infected individuals and addressed the question of whether this approach can define bona fide groups of slow and rapid responders to ART. First, using the third quartile Q3 of the decline rate as a parameter, we found that the frequency of $TIM\text{-}3^+$ pDCs detected in HIV-1-infected individuals before ART (Figure 3D,E) and in the same patients over the 3-month ART (Figure 3F) was significantly higher in slowly responding ($[VL_{0\text{-mo}}/VL_{3\text{-mo}}]$ $\log_{10}$ < Q3) than in rapidly responding ($[VL_{0\text{-mo}}/VL_{3\text{-mo}}]$ $\log_{10}$ > Q3) individuals ($p = 0.015$ before ART, $p = 0.012$ after 3-month ART). TIM-3 was the only phenotypic marker analyzed in this study that correlated with the rate of decline in plasma HIV-1 RNA copies/mL. Then, we analyzed correlation of the frequency of $TIM\text{-}3^+$ pDCs in the whole cohort of 21 HIV-1-infected individuals with the rate of decline in plasma HIV-1 RNA (Figure 3G,H). In both HIV-1-infected individuals before ART (Figure 3G) and in the same patients over the 3-month ART (Figure 3H), the rate of decline in HIV-1 RNA level significantly correlated with the frequency of $TIM\text{-}3^+$ pDCs. In contrast, correlation of the total HIV-1 RNA level ($VL_{0\text{-mo}}$ $\log_{10}$ copies/mL) before ART with the frequency of $TIM\text{-}3^+$ pDCs was not significant ($p = 0.44$).

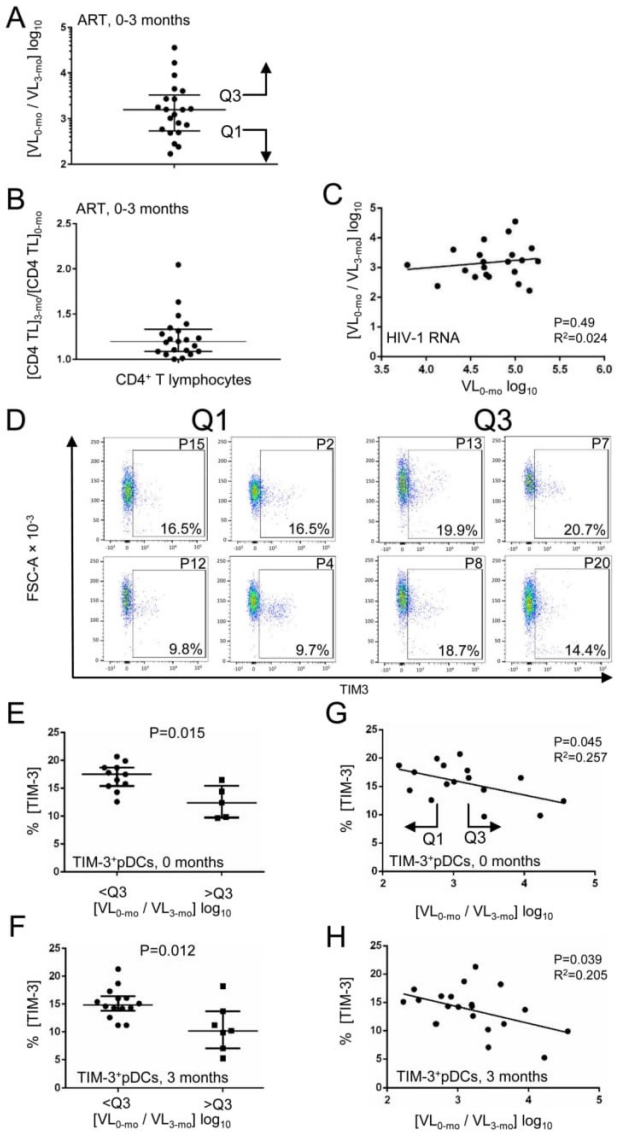

Figure 3. Expression of TIM-3 on pDCs of HIV-1-infected individuals negatively correlates with the rate of decline in HIV-1 RNA copies/mL of plasma over the 3-month ART. (**A**) The rate of decline in HIV-1 virus load (VL) expressed as $[VL_{0\text{-mo}}/VL_{3\text{-mo}}]\ \log_{10}$, where $VL_{0\text{-mo}}$ 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. (**B**) The recovery rate of $CD4^+$ T cells in HIV-1-infected individuals expressed as a ratio of CD4 T cell number after 3 months of ART $[CD4\ TL]_{3\text{-mo}}$ to CD4 T cell number at zero time of ART $[CD4\ TL]_{0\text{-mo}}$. (**C**) 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). (**D**) Examples of dot plots for the quantification of TIM3 in $Lin^-BDCA\text{-}2^+$-gated live pDCs are shown for PBMCs from treatment-naïve individuals from Q1 (patients P2, P4, P12, P15) and Q3 (patients P7, P8, P13, P20). (**E**,**F**) Comparison of the frequency of TIM-3^+ pDCs in treatment-naïve HIV-1-infected patients (**E**) or patients after the 3-month ART (**F**), in which $[VL_{0\text{-mo}}/VL_{3\text{-mo}}]\ \log_{10}$ was <Q3 or >Q3. (**G**,**H**) Correlation of the frequency of TIM-3 expressed on pDCs in treatment-naïve HIV-1-infected patients (**G**) or patients after the 3-month ART (**H**) with $[VL_{0\text{-mo}}/VL_{3\text{-mo}}]\ \log_{10}$ (the same samples as in panels (**E**,**F**) were analyzed). 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.

4. Discussion

Although multiple markers of active immune state are significantly reduced by ART, immune activation following sustained suppression of HIV-1 RNA in plasma remains significantly elevated when compared to uninfected controls [22–26]. Using a cohort of patients who were subjected to multiple samplings before and 3 and 9 months after ART initiation, we demonstrated partial restoration of adaptive immune function, as evidenced by the increase in the average number of $CD4^+$ T cells and B cells to standard levels of healthy donors [27–29]. In contrast, the average numbers of NK cells ($CD3^-/CD16^+/CD56^+$) and $CD8^+$ T cells remained significantly higher over the standard reference ranges in healthy individuals [27–29].

Our results show that over a period of 9-months, ART partially restored pDC numbers, however, the immunogenic phenotype of pDCs was not significantly restored. While impairment of single pDC functions was demonstrated in several reports [15,18,19,30,31], in the present study we analyzed a complex dysfunction of pDC covering expression of the MHC class II ligand, the high affinity HIV-1 receptor CD4, the regulatory receptor BDCA-2, the Fcγ receptor CD32, the pDC dysfunction marker TIM-3, and the marker of killer pDC, TRAIL.

We found that TIM-3 was the only phenotypic markers among the pDC markers analyzed in this study whose expression correlated with the rate of decline in HIV-1 RNA level after the initiation of ART. Correlation of the rate of decline in HIV-1 RNA copies with the TIM-3 expression level at both time 0 and over 3 months of ART is consistent with sustained expression of TIM-3 during ART. We suggest that the rate of decline in HIV-1 RNA level during the first period after initiation of ART, in our case 3 months, could be a new additional parameter to characterize response to ART. A higher number of enrolled patients and a longer follow-up period will be necessary to evaluate the possible clinical significance of this parameter.

Several immune mechanisms participate in clearance of HIV-1 during ART. Among them, impaired production of IFN-α and TNF-α regulated in pDCs by TIM-3 can play an important role in the immunopathogenesis of HIV-1 infection [18]. During HIV-1 infection, pDC-activating TLR7/9 agonists induce TIM-3 expression and subsequently result in the impairment of pDC function. It was shown that IFN-α and TNF-α production was impaired in $TIM\text{-}3^+$ pDCs, and that TIM-3 may transfer TLR agonists into acidic lysosomes, bypassing TLR activation [18]. The high level of colocalization of TIM-3 and IRF7 within lysosomes also suggests a mechanism by which TIM-3 may regulate IFN-α production.

The molecular mechanism of depletion of circulating pDCs accompanied by the impaired secretion of IFN-I and proinflammatory cytokines and capacity of antigen presentation remains elusive despite years of intense research [4–6,20,32–35]. HIV-1-exposed pDCs express an increased level of markers of pDC dysfunction, as PD-L1, PD-L2, TIM-3, and BDCA-2. The increased level of the median value of MFIs of BDCA-2 in pDCs of HIV-1-infected individuals in comparison to healthy donors is of special interest. In contrast to MFI, the frequency of $BDCA\text{-}2^+$ pDCs is not influenced by HIV-1 infection, and in vitro activation of pDC via TLR7/9 agonists leads to downregulation of the pDC surface-localized BDCA-2 [14]. Thus, some other signals should be responsible for increased expression level of BCDA-2 on pDCs of HIV-1-infected individuals. Signaling via pDC regulatory receptors, including BDCA-2, attenuates TLR-induced production of IFN-I and proinflammatory cytokines [4–6,20,33,35–37]. Although by different mechanisms, TIM-3 also inhibits production of IFN-α and TNF-α in pDCs. IFN control is hijacked in the pathogenesis of several chronic viral infections including HIV-1, leading to immune tolerance [6,21,38,39].

5. Conclusions

We conclude that the immunogenic phenotype of pDCs is only partially restored after sustained suppression of HIV RNA level in ART-treated patients and that a high level of pDC-expressed TIM-3 in treatment naïve patients could be a useful predictive biomarker of a slow decline in HIV-1 RNA level during ART. Establishing the clinical significance and generalizability of this observation will require larger numbers of patients and more extended follow-ups with clinical outcomes.

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Toll-like receptor dual-acting agonists are potent inducers of PBMC-produced cytokines that inhibit hepatitis B virus production in primary human hepatocytes

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Recombinant interferon-α (IFN-α) treatment functionally cures chronic hepatitis B virus (HBV) infection in some individuals and suppresses virus replication in hepatocytes infected in vitro. We studied the antiviral effect of conditioned media (CM) from peripheral blood mononuclear cells (PBMCs) stimulated with agonists of Toll-like receptors (TLRs) 2, 7, 8 and 9. We found that CM from PBMCs stimulated with dual-acting TLR7/8 (R848) and TLR2/7 (CL413) agonists were more potent drivers of inhibition of HBe and HBs antigen secretion from HBV-infected primary human hepatocytes (PHH) than CM from PBMCs stimulated with single-acting TLR7 (CL264) or TLR9 (CpG-B) agonists. Inhibition of HBV in PHH did not correlate with the quantity of PBMC-produced IFN-α, but it was a complex function of multiple secreted cytokines. More importantly, we found that the CM that efficiently inhibited HBV production in freshly isolated PHH via various cytokine repertoires and mechanisms did not reduce covalently closed circular (ccc)DNA levels. We confirmed our data with a cell culture model based on HepG2-NTCP cells and the plasmacytoid dendritic cell line GEN2.2. Collectively, our data show the importance of dual-acting TLR agonists inducing broad cytokine repertoires. The development of poly-specific TLR agonists provides novel opportunities towards functional HBV cure.

Chronic infection with hepatitis B virus (HBV) is a major public health problem affecting approximately 250 million people worldwide. Despite a weak innate immune response to HBV approximately 90% of adults clear HBV, presumably via the induction of an effective CD8 + T cell response[1–3]. Treatment of chronic hepatitis B with nucleot(s)ide analogues inhibits the formation of new infectious viral particles but does not eliminate stable covalently closed circular DNA (cccDNA) in hepatocytes. Pegylated interferon α (IFN-α) treatment can be

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considered as an alternative therapy for people with mild-to-moderate chronic hepatitis B[4]. However, in addition to causing undesired side effects, IFN-α monotherapy leads to functional cure in less than 8% of people with chronic hepatitis B[4–7].

Results from cell culture experiments have demonstrated that type I IFNs (IFN-I, IFN-α, β, ε, ω) as well as type III IFNs (IFN-III, IFN-λ1, 2, 3) affect HBV cccDNA either directly through epigenetic transcriptional silencing[8] or by reducing its stability[9–11]. In HBV-infected hepatocytes, IFN-I induces hundreds of IFN-stimulated genes (ISGs) that restrict HBV infection at different levels[12]. IFN-α induces soluble factors that inhibit HBV entry into cells[13], protein kinase R, which inhibits HBV protein translation[14], and tetherin, which blocks release of HBV from infected hepatocytes[15]. A side-by-side comparison of a large panel of cytokines in vitro revealed that pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β and IL-6, are as efficient as IFNs at inhibiting HBV replication[16–18]. Thus, both IFNs and proinflammatory cytokines control HBV replication and contribute to HBV cure in different models[17,19].

The lack of curative anti-HBV therapies highlights the potential importance of different immune-modulators and their agonists[20–24]. Among agonists of pattern recognizing receptors expressed in primary liver cells[25], namely agonists of Toll-like receptors (TLRs) attracted interest because of their potency to induce IFNs and proinflammatory cytokines and chemokines in both hepatocytes and non-parenchymal cells[23,24]. Moreover, it was shown that GS-9620 (vesatolimod), an agonist of endosomal TLR7, which is preferentially expressed in plasmacytoid dendritic cells (pDCs)[26–30] but not in primary hepatocytes (PHH)[23,31,32], significantly reduced viremia and cccDNA expression, and led to functional cure in animal models[20–22]. A recent study showed that TLR1/2 and TLR3 ligands inhibit HBV replication in PHH, and that the same ligands also induce the production of antiviral cytokines in peripheral blood mononuclear cells (PBMCs)[23]. Another study showed that inhibition of HBV replication in PHH could be mediated by conditioned media (CM) from PBMCs stimulated with GS-9620[24]. IFN-I secreted by TLR7-agonist-stimulated PBMCs was identified as the major substance inhibiting HBV production without reducing cccDNA levels[24].

Several studies demonstrated that combination of different TLR agonists or a single TLR agonist with other immune-modulators potentiated the immunotherapeutic effect[33,34]. However, the effect of poly-specific TLR agonists like recently developed TLR2/7 dual-acting agonist CL413 (Adilipolin), a chimeric molecule that co-activates the cell surface receptor TLR2 and the endosomal receptor TLR7[35], on HBV infection was not elucidated. Here, we compared the antiviral effect of CM from PBMCs stimulated with dual-acting agonists with the effect of CM from PBMCs stimulated with agonists for single TLR. We found that CM from PBMC stimulated with a dual-acting agonist of TLR7/8 (R848) and TLR2/7 (CL413) were more potent drivers of inhibition of hepatitis *e* and *s* antigens (HBeAg and HBsAg) production from HBV-infected PHH than CM from PBMCs stimulated with agonists specific only for TLR7 (GS-9620, CL264) or TLR9 (CpG-A, CpG-B). Inhibition of HBV in PHH did not correlate with the level of PBMC-produced IFN-α, but it was a complex function of multiple secreted cytokines. We addressed the question whether CM, which efficiently inhibited the production of HBV in PHH via different repertoires of cytokines would also reduce the cccDNA levels.

Results

Differential potency of TLR agonists in the induction of PBMC-secreted cytokines. First, we determined the levels of selected cytokines secreted into supernatants (conditioned media, CM) of PBMCs stimulated for 16 h by different agonists of TLR7 (CL264-CM, GS-9620[L]-CM (50 nM)), TLR7/8 (R848-CM, GS-9620[H]-CM (10 μM)), TLR9 (CpG-A-CM, CpG-B-CM) and a TLR2/7 dual agonist (CL413-CM) (Fig. 1, linear plot, Supplementary Fig. S1, logarithmic plot). Two concentrations of GS-9620 were used: at a low concentration (GS-9620[L], 50 nM) it shows a high selectivity for activation of TLR7 over TLR8[36], while a higher concentration (GS-9620[H], 10 μM) elicits combined TLR7 and TLR8 stimulation. Among the cytokines present in CM, we quantified those previously shown to regulate HBV replication, including type I, II and III IFNs (IFN-α, γ, λ); the proinflammatory cytokines TNF-α, IL-6 and IL-12; the chemokine IL-8; and the regulatory cytokine IL-10[8–11,13–15]. While IFN-α and IFN-λ1 were predominantly induced by CpG-A, the proinflammatory cytokines IFN-γ, TNF-α, IL-6, IL-8 and IL-12 were predominantly induced by R848. IL-6, IL-8 and IL-12 were also significantly stimulated by CL264-CM, GS-9620[H]-CM and CL413-CM. The latter agonists also stimulated production of the anti-inflammatory cytokine IL-10. Then, we determined by dynamic phospho-flow cytometry phosphorylation of the NF-κB p65 subunit in PBMCs exposed for 1 h to different TLR agonists (Supplementary Fig. S2)[37]. Stimulation for this time interval, which was insufficient for cytokine production, resulted in phosphorylation of p65 NF-κB in PBMCs exposed to dual-acting agonists R848 (20.3%), CL413 (20.8%) and GS-9620[H] (6.3%). In contrast, the single-acting agonists, GS-9620[L] (0.6%) and CpG-A (0.6%), did not induce the NF-κB p65 phosphorylation. In summary, PBMCs stimulated by different TLR2/7, TLR7, TLR7/8 and TLR9 agonists produced broad and variable repertoires of type I, II and III IFNs and proinflammatory cytokines.

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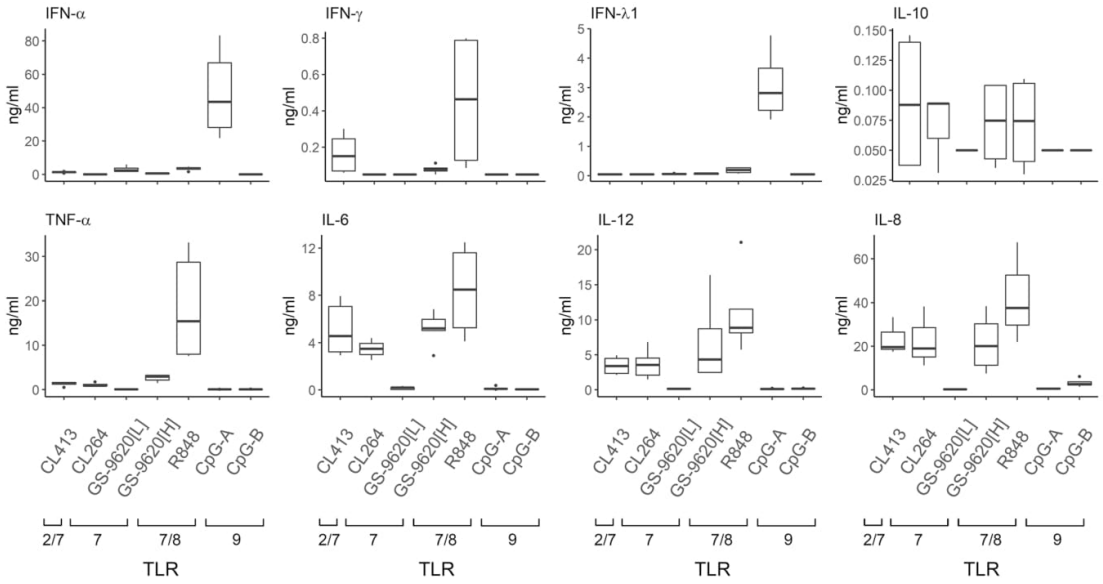

Figure 1. Cytokines secreted by PBMCs stimulated by different TLR2/7, TLR7, TLR7/8 and TLR9 agonists. PBMCs (N > 3) were stimulated with the TLR2/7 dual-agonist CL413 (5 µg/ml), the TLR7 agonists CL264 (5 µg/ml) and GS-9620[L] (50 nM), the TLR7/8 agonists GS-9620[H] (10 µM) and R848 (4 µg/ml), and the TLR9 agonist CpG-A (4 µg/ml) or CpG-B (4 µg/ml) for 16 h, and the cytokine levels were determined by ELISA. The data are shown as medians and interquartile ranges. See Supplementary Fig. S1 for logarithmic plot.

IL-12, than with GS-9620[L]-CM. Within the variable repertoires of IFNs and proinflammatory cytokines, the levels of IFN-α and IFN-λ1 and the levels of the proinflammatory cytokines and chemokines tested—IFN-γ, TNF-α, IL-6, IL-8 and IL-12—correlated across the agonists evaluated ($R \geq 0.7$) (Supplementary Table S3). More importantly, HBeAg levels negatively correlated with the quantity of IFN-γ, TNF-α, IL-6, IL-8 and IL-12 ($R \geq 0.7$). Taken together, analysis of HBeAg production revealed that inhibitory levels do not correlate with the quantity of secreted IFN-α when other antiviral cytokines like IL-6, TNF-α and IFN-γ are produced by PBMCs. Our data support a model where not a single cytokine, but a complex function of multiple PBMC-secreted cytokines is associated with CM-mediated HBV inhibition in PHH.

Total HBV DNA, but not cccDNA, in HBV-infected PHH is reduced by CMs from TLR2/7, TLR7, TLR7/8, and TLR9 agonist-stimulated PBMCs. Treatment of freshly isolated HBV-infected PHH with CpG-A-CM, GS-9620[L]-CM, GS-9620[H]-CM or R848-CM or treatment with 1,000 IU of recombinant IFN-α or IFN-λ led to an approximately 50% reduction in intracellular HBV DNA levels (Fig. 3A). No decrease in cccDNA was detected in the same DNA samples from three PHH donors by qPCR using specific cccDNA primers (Kruskal–Wallis $p = 0.443$) (Fig. 3B). In addition, we used qPCR to evaluate the effect of the dual TLR agonists-induced R848-CM and GS-9620[H]-CM on HBV cccDNA in PHH from one donor. However, CM from PBMCs stimulated by these dual TLR agonists also did not reduce the cccDNA level. Moreover, we used droplet-digital (dd)PCR to verify the effect of TLR dual agonists, including GS-9620[H]-CM or R848-CM and CL413-CM, on cccDNA and to assess the quality of cccDNA sample preparation (Supplementary Table S4). Data obtained by ddPCR confirmed the importance of T5 exonuclease treatment and selection of cccDNA-specific primers. Collectively, our results suggest that none of the selected TLR agonists reduced cccDNA in our in vitro PHH culture system.

Coculturing with stimulated PBMCs inhibits HBV production from PHH. To test whether continuous production of cytokines from TLR2/7, TLR7, or TLR9 agonist-stimulated PBMCs inhibits the production of HBeAg from HBV-infected PHH more strongly than two-times addition of CM to infected cells, we cocultured TLR agonist-stimulated PBMCs with HBV-infected PHH in the Transwell system from 6–9 DPI (Fig. 4A). We found that inhibition of HBeAg in HBV-infected PHH by coculture with TLR2/7 (CL413), TLR7 (GS-9620[L]) or TLR9 (CpG-A) agonist-stimulated PBMCs did not significantly differ from inhibition following two-times addition of CM (Fig. 4B). In any case, inhibition did not exceed 70%, and CL413 was a more potent inducer of an antiviral response than CpG-A or GS-9620[L]. Previous study found that several TLR agonists can inhibit HBV replication both directly via TLR activation in PHH and indirectly via exposure to CM of stimulated innate immune cells[23]. Thus, we tested whether the TLR2/7 dual agonist CL413 can inhibit HBV replication without the indirect effect of PBMC-secreted cytokines (Fig. 4B). However, in the absence of PBMCs, CL413 did not show any antiviral activity, although it induced production of proinflammatory cytokines IL-6 (275 pg/ml), TNF-α (84 pg/ml) and chemokine IL-8 (987 pg/ml) in HBV-infected PHH. As in the case of CM addition to HBV-infected cells, coculture of TLR agonist-stimulated PBMCs with HBV-infected PHH in the Transwell system did not result in degradation of cccDNA (data not shown).

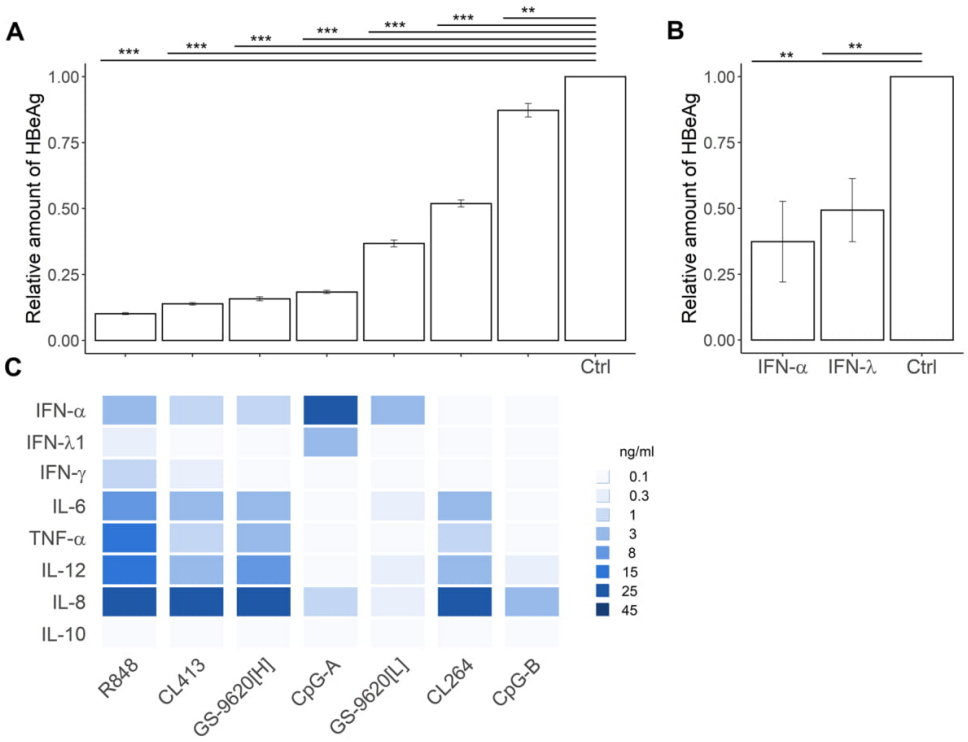

Figure 2. Inhibition of HBeAg production from HBV-infected PHH treated with PBMC CM. (**A**) A total of 65,000 PHH were infected with 500 viral genome equivalents (VGE) of HBV per cell and cultured for 3 days before conditioned medium (CM, diluted 1:10) was added. CM was derived from 3×10^6 PBMCs per ml stimulated by agonists of TLR2/7 (CL413), TLR7 (CL264, GS-9620[L]), TLR7/8, (R848, GS-9620[H]), or TLR9 (CpG-A, CpG-B) for 16 h. CM was added again 6 days post-infection (DPI). Production of HBeAg was determined by ELISA 9 DPI and normalized to production by HBV-infected PHH in the absence of CM. The HBeAg data are shown as mean ± SEM from five independent experiments with PHH from three donors (N = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ pairwise Wilcoxon test. Kruskal–Wallis $p < 2.2 \times e^{-16}$. (B) HBV-infected PHH treated with 1,000 IU/ml of recombinant IFN-α-2a or IFN-λ3. The data are shown as mean ± SEM from three independent experiments with PHH from two donors (N = 2). $^{**}p < 0.01$, Mann–Whitney–Wilcoxon pairwise test, p value adjusted by Benjamini–Hochberg (BH) method. (**C**) Quantity of cytokines in CM from stimulated PBMCs plotted as a heat diagram representing the median values that is shown in Fig. 1.

Anti-IFN-I receptor monoclonal antibody (IFNAR mAb) abrogates CpG-A-CM or GS-9620[L]-CM-induced inhibition of HBeAg production from HBV-infected PHH. Subsequently, we investigated the mechanism of pDC-induced inhibition of HBeAg production in HBV-infected PHH. Due to the sensitivity of HBeAg production in HBV-infected PHH to IFN-α and IFN-λ, we examined the proportion of the inhibitory effect mediated by type I and III IFN receptors, IFNAR and IFNLR (Fig. 5A). To do so, we pretreated HBV-infected PHH with mAbs targeting IFNAR and IFNLR and determined the level of HBeAg produced upon exposure of HBV-infected PHH to CpG-A-CM or GS-9620[L]-CM (Fig. 5B). While IFNAR mAb completely abrogated the inhibitory effect of GS-9620-CM, it abrogated by only 40% the inhibitory effect of CpG-A-CM. Simultaneous blockade of IFNAR and IFNLR did not significantly increase abrogation of the inhibitory effect on HBeAg production. Production of HBeAg was also significantly inhibited by 1,000 IU of recombinant IFN-α2a (by 61.3%, $p = 0.04$) and IFN-λ3 (by 56.2%, $p = 0.04$).

Stimulated GEN2.2 pDCs show antiviral activity against HBV-infected HepG2-NTCP cells. We next compared the antiviral effect of CM from stimulated PBMCs on virus production in PHH (Fig. 2) with that in a model comprising GEN2.2 pDCs and HBV-infected HepG2-NTCP hepatocytes[26,38,39] (Fig. 6A). To facilitate 4 days lasting coculture, which is still difficult to perform in rare and in vitro short living human primary pDCs, we performed our studies in human pDC line GEN2.2, which shares many features with human primary pDCs[26,39,40]. Production of HBeAg from HBV-infected HepG2-NTCP hepatocytes was significantly inhibited by exposure to CM from GEN2.2 cells stimulated with CpG-A (by 67%), CpG-B (by 59%), GS9620[L] (by 55%) and GS-9620[H] (by 53%) (Fig. 6B). No significant differences in inhibition of HBeAg production were observed when the antiviral effect of CM from stimulated GEN2.2 cells was compared with direct coculture of GEN2.2 and HepG2-NTCP cells (Fig. 6C). Despite the different repertoires and levels of cytokines induced by CpG-A

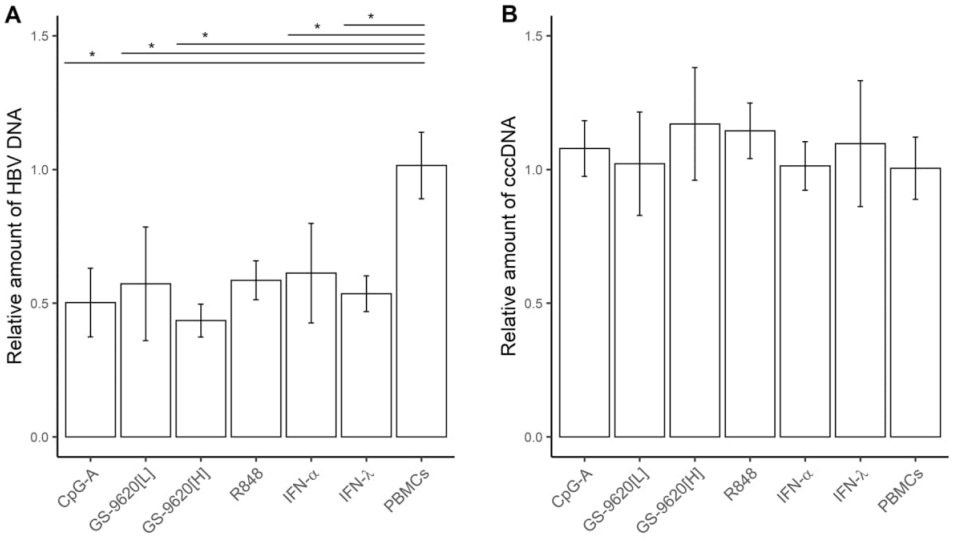

Figure 3. Reduction of total HBV DNA (**A**) but not cccDNA (**B**) in HBV-infected PHH by CM from TLR agonist-stimulated PBMCs. PHH were infected with HBV and cultured for 3 days followed by addition of CpG-A-CM, GS-9620[L]-CM, GS-9620[H]-CM or R848–CM (diluted 1:10) or 1,000 IU of IFN-α or IFN-λ. CM was added again 6 DPI. Cells were grown for 3 more days and the quantities of total HBV DNA and cccDNA were determined by qPCR. Data are shown as mean ± SEM with PHH from three donors (N = 3) for CpG-A-CM, GS-9620[L]-CM, and with PHH from one donor (N = 1) for GS-9620[H]-CM, R848-CM (two biological replicates) $*p < 0.05$, Dunn's test, p value adjusted by Benjamini-Hochberg (BH) method.

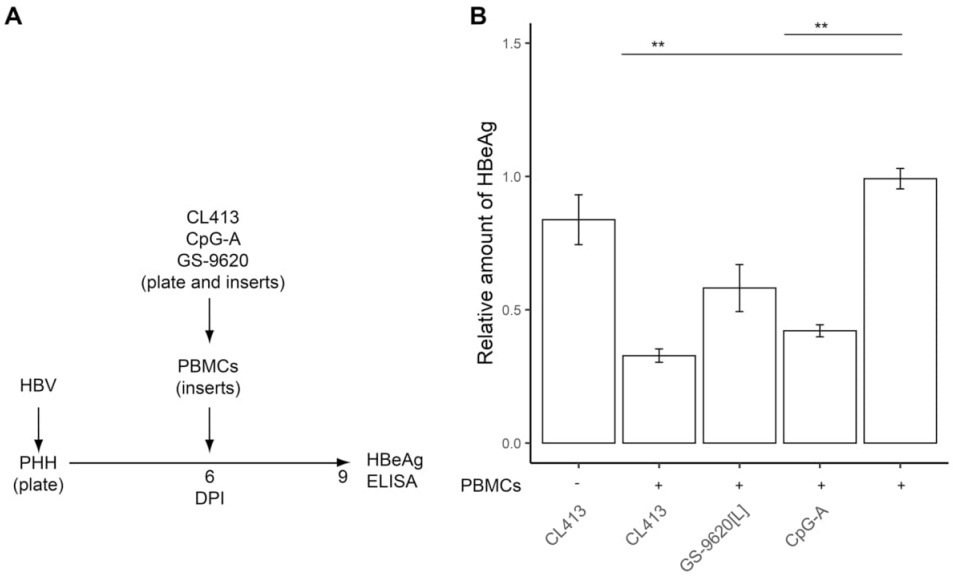

Figure 4. Inhibition of HBeAg production from HBV-infected PHH by coculture with TLR2/7, TLR7 or TLR9 agonist-stimulated PBMCs. (**A**) Experimental flow chart. PHH were infected with HBV and kept in culture for 6 days before a Transwell insert containing PBMCs stimulated with the TLR2/7 agonist CL413 (5 µg/ml), TLR9 agonist CpG-A (4 µg/ml), or TLR7 agonist GS-9620[L] (50 nM) was added. (**B**) Production of HBeAg was normalized to production of HBV-infected PHH cells cocultured with non-stimulated PBMCs. In one parallel, a culture of HBV-infected PHH was exposed to CL413 in the absence of PBMCs. Data are shown as mean ± SEM of three biological replicates with PHH from two donors (N = 2). $**p < 0.01$, Mann–Whitney–Wilcoxon pairwise test, p value adjusted by BH method.

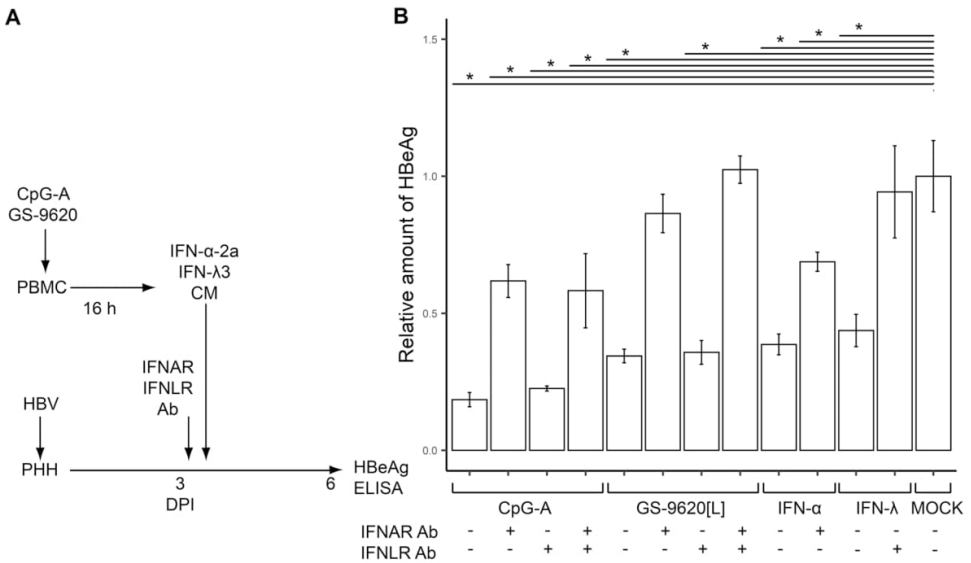

Figure 5. IFNAR mAb abrogates inhibition of HBeAg production from HBV-infected PHH by CpG-A-CM or GS-9620[L]-CM. (**A**) Experimental flow chart. HBV-infected PHH were exposed at 3 DPI to 5 µg/ml of IFNAR mAb, IFNLR mAb or control isotype mAb. Recombinant IFN-α-2a or IFN-λ3 (1,000 IU/ml) was used as a control. Production of HBeAg was determined by ELISA 6 DPI. (**B**) Production of HBeAg was normalized to production by HBV-infected PHH treated with CM from unstimulated PBMCs determined by ELISA 6 DPI. The data are shown as mean ± SEM from three independent experiments with PHH from one donor (N = 1). *$p < 0.05$, Mann–Whitney–Wilcoxon pairwise test, p value adjusted by BH method.

(50 ng/ml IFN-α), CpG-B (4 ng/ml IFN-α), GS-9620[L] (50 ng/ml IFN-α), and namely GS-9620[H] (< 50 pg/ml IFN-α) (Fig. 6D), all three agonists similarly inhibited HBeAg (by approximately 65%) (Fig. 6B,C).

Discussion

In this study, we investigated the antiviral effect of CM from PBMCs stimulated with a set of agonists of endosome-localized TLRs. Our results demonstrate that synthetic TLR agonists capable of activating more than one TLR induce a broader proinflammatory cytokine spectrum and are more efficient drivers of HBV inhibition than single TLR-targeting agonists. The dual-acting TLR agonists R848[41], CL413[35] and GS-9620[H][36] were the best-scoring inducers of HBV inhibition. Statistical significance was a major issue in these experiments, which were performed in fresh PHH from 3 donors, each tested in 5 biological replicates. None of the selected TLR ligands reduced the level of cccDNA in HBV-infected cells. Previous findings revealed that R848 can be independently recognized by both human TLR7 and TLR8, although TLR8 is induced more efficiently than TLR7 at higher R848 concentrations[41]. A low concentration of GS-9620[L] has approximately 30-fold higher selectivity for activation of TLR7 over TLR8, with no detectable activity on other human TLRs[36]. However, GS-9620 elicits combined TLR7 and TLR8 stimulation at higher concentrations[36]. As TLR7 expression is largely restricted to pDCs in PBMC subsets[26–30,42] and TLR8 is predominantly expressed in myeloid DCs and monocytes[43,44], we surmise that low concentrations of GS-9620[L] (50 nM) preferentially mediate secretion of IFN-α from pDCs, whereas high concentrations of GS-9620[H] (10 µM) preferentially stimulate secretion of inflammatory cytokines in classical myeloid DCs and CD14+ monocytes.

Another dual-acting agonist, CL413, also induces both IFN-I and proinflammatory cytokines. We tested CL413 activity to analyze both a direct effect on TLR2 stimulation in PHH and an indirect effect on TLR7 stimulation in PBMCs. CL413 did not elicit a direct inhibitory effect on HBV replication in TLR2-expressing PHH. Therefore, its antiviral effect likely is conferred indirectly by triggering TLR2 and TLR7 in PBMCs. Expression of cytoplasmic TLR2 and endosome-localized TLR7 in CD14+ monocytes permits both signaling pathways to be triggered at the single-cell level[44]. In contrast, in pDCs in which TLR2 is not expressed, only TLR7 signaling can be activated by CL413. Recently, the dual-acting agonist Riboxxol, which triggers TLR2/3-mediated signaling via the IFN or NF-κB pathways, was shown to efficiently and directly suppress HBV replication in HBV-infected PHH[23]. In contrast to dual-acting agonists, single-acting ligands of TLR7 (CL264) or TLR9 (CpG-B) induced in PBMCs only moderate levels of proinflammatory cytokines with no detectable IFN-I, and CL264-CM and CpG-B-CM were associated with poor HBeAg inhibition. Robust production of proinflammatory cytokines induced in PBMCs by dual-acting agonists was associated with elevated phosphorylation of p65 NF-κB.

To induce a large and variable range of cytokines, we stimulated PBMCs with a larger spectrum of agonists than those used in previous studies[23,24]. Our results indicate that inhibition of HBeAg and HBsAg production

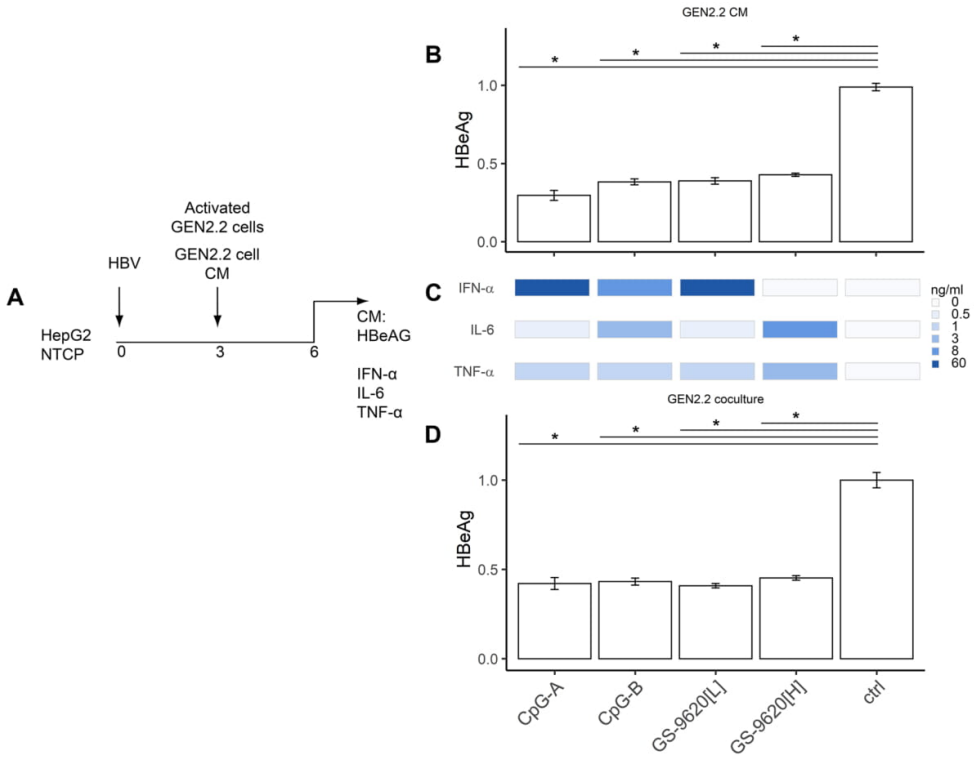

Figure 6. Antiviral activity of stimulated GEN2.2 pDCs on HBV-infected HepG2-NTCP cells. (**A**) Experimental flow chart. A total of 60,000 HepG2-NTCP cells were infected with 2000 VGE per cell of HBV and cultured for 3 days before CM from GEN2.2 cells stimulated with CpG-A (4 µg/ml), CpG-B (4 µg/ml), or GS-9620[L or H] (50 nM or 10 µM) was added (**B**). Levels of IFN-α, IL-6 and TNF-α (**D**) in CM were determined by ELISA 6 DPI. HBV-infected HepG2-NTCP cells were cocultured with 100,000 stimulated GEN2.2 cells (**C**). Production of HBeAg was normalized to production by HBV-infected HepG2-NTCP cells in the absence of pDCs. The data are shown as means ± SEM from three independent experiments. $^{*}p < 0.05$, Mann–Whitney–Wilcoxon pairwise test, p value adjusted by BH method.

in HBV-infected PHH does not correlate with the quantity of PBMC-secreted IFN-α, but rather is a complex function of multiple secreted cytokines. We found that CpG-A-activated PBMCs produced more IFN-I and IFN-III than those stimulated with GS-9620[L], which has been tested extensively in previous HBV inhibition-related studies[20–24]. In agreement with the previous finding that IFN-I is the major component of PBMC CM responsible for inhibition of HBV production in PHH, CpG-A-CM inhibited HBeAg and HBsAg secretion more efficiently than GS-9620[L]. However, a higher concentration of GS-9620[H] (10 µM), which induced in PBMCs a broader spectrum of proinflammatory cytokines but a lower quantity of IFN-I than induced by GS-9620[L], was associated with significantly higher HBeAg and HBsAg inhibition. R848, which also induced a very broad spectrum of proinflammatory cytokines, had a similar antiviral effect as GS-9620[H]. Importance of the cytokine complexity in inhibition of HBV production is further highlighted by relatively low inhibitory activity (50 to 60%) of recombinant IFN-α-2a and IFN-λ3. Based on the blockade of IFNAR by mAb, Lucifora et al.[23] concluded that the antiviral effect of TLR1/2 and TLR3 activation in PHH was not due to type-I IFN and IL-6 production. Correlation analysis showed that in addition to IFN-α, the proinflammatory cytokines IL-6, TNF-α and IL-12 and the chemokines IL-8 and IFN-γ are major contributors to anti-HBV inhibitory activity. A statistical model that could decipher the specific combination of cytokines necessary to inhibit HBV production from infected hepatocytes would require additional measurements of the effect of CM or artificial permutations of recombinant cytokines.

Although R848-CM, CL-413-CM, CpG-A-CM and GS-9620[H]-CM achieved two to fourfold greater inhibition of HBeAg than that observed with GS-9620[L]-CM in previous studies[23,24], none resulted in reduction of cccDNA levels in freshly isolated PHH. This is compatible with recent findings showing that GS-9620[L]-CM strongly induces various IFN-stimulated genes and inhibits virus production in HBV-infected PHH without inducing APOBEC3A or the Smc5/6 complex—and without reducing cccDNA levels[24]. The importance of cccDNA and its degradation for HBV cure positions this molecule in the center of HBV research. A 2012 study reported that IFN-α inhibits cccDNA transcription by hypoacetylation of cccDNA-bound histones and reduces binding of the STAT1 and STAT2 transcription factors to the IFN-stimulated response element present in the

HBV genome[8]. More recent studies have shown that cccDNA can be degraded in HBV-infected hepatocytes in a noncytopathic fashion during IFN-α treatment[9,10].

Production of HBeAg from the HBV-infected hepatoma cell line HepG2-NTCP was three- to fivefold less sensitive to IFN-α compared to production from HBV-infected PHH. In the presence of CM or recombinant IFN-I, residual production of HBeAg in HepG2-NTCP cells was not suppressed below 35%. The insignificant differences in inhibition of HBV production by exposure of HBV-infected HepG2-NTCP cells directly to activated GEN2.2 cells or to their CM indicate that soluble factors, and not cell-to-cell contact during coculture, plays a major role in the regulation of HBV production.

We also addressed whether IFN-I and IFN-III present in CpG-A-CM cooperate in HBV inhibition, which could explain the greater inhibitory effect of CpG-A-CM compared to GS-9620[L]-CM. Surprisingly, IFNLR targeting had no effect on HBeAg secretion, and only IFNAR mAb partially abrogated the inhibitory effect of CpG-A-CM. This inhibitory effect was not completely reverted by targeting both IFNAR and IFNLR, likely due to inefficient inhibition of IFNAR. Thus, we cannot conclude whether IFN-I is the main inhibitory driver in CpG-A-CM or if other cytokines contribute as well. Further study will be necessary to elucidate whether IFN-I signaling dominates over IFN-III signaling in PHH.

Specific and prolonged suppression of chronic hepatitis B in chimpanzee and woodchuck models by endosomal TLR7, 8, and 9 agonists led to an interest in discerning the mechanisms by which these TLR ligands elicit antiviral responses[20–22]. However, clinical studies with GS-9620, which showed the best antiviral effect in animal models, did not reveal a clinically significant decline of HBsAg at tolerable doses (two one-weekly doses 4 mg) in patients with chronic hepatitis B[45,46]. This dose corresponds to concentration of 4.6 ng/ml in plasma, while 50 ng/ml of GS-9620[L] were used in our experiments[47]. The dose of CpG ODNs commonly used in clinical trials was 1.5–15 µg/kg and the schedule of ODN administration ranged from weekly to monthly. In preclinical studies, much higher doses of CpG ODN (2.5 mg/kg) were administered daily to mice while 4 µg/ml of CpG-A or CpG-B were used in our experiments[48]. Also the dose of CL413 agonist (5 µg/ml) was within the range of the dose administered to mice in preclinical studies (3 µg/g)[49]. Further studies of the distinct antiviral potential of polyspecific TLR agonists are necessary to elucidate their functions, which could provide new opportunities for the development of novel strategies to achieve sustained viral clearance and provide a definitive cure for hepatitis B.

Methods

Hepatocyte cell cultures. HepG2-NTCP (a human liver cancer cell line, HepG2, stably transfected with the human HBV entry receptor—sodium taurocholate cotransporting polypeptide [hNTCP]) was obtained from Dr. Stephan Urban of Heidelberg University Hospital, Heidelberg, Germany. HepG2.2.15 (a HepG2 cell line that harbors two head-to-tail dimers of the HBV genome [serotype ayw, genotype D; GenBank accession: U95551.1]) was obtained from Dr. David Durantel of the Cancer Research Center of Lyon, Lyon, France. These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and puromycin (0.05 mg/ml) or G418 (0.4 mg/ml), respectively[50]. HepAD38 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and tetracycline (0.3 µg/ml). Primary human hepatocytes (PHHs) were isolated from liver resections as described by David et al.[51]. Briefly, liver biopsies were first perfused with Hanks Balanced Salt Solution (HBSS) lacking Ca^{2+} and supplemented with 0.5 mM EGTA (Merck). Then, the liver tissue fragments were perfused with HBSS supplemented with Ca^{2+} and 0.05% Collagenase (Merck). The liver cell suspension was filtered through a 100 µm Cell Strainer (Corning), centrifuged at 50 × g for 3 min at 4 °C and washed 3 times with L-15 Medium (Thermo Fischer Scientific). Cell viability was estimated by trypan-blue exclusion, and the cells were seeded on collagen-coated plates. PHHs were maintained in Williams E Medium (Thermo Fisher Scientific) supplemented with the Primary Hepatocyte Maintenance Supplement Kit (Gibco).

PBMCs and GEN2.2 cell line. PBMCs were isolated and cultured as previously described[37,52]. The GEN2.2 cell line was cultured with mouse MS5 cell line in RPMI 1,640 supplemented with 10% FBS[40].

Inhibitors, antibodies and reagents. CpG-A (ODN 2,216), CpG-B (ODN 2006), CL413 (Adilipolin), CL267 and R848 were obtained from InvivoGen (San Diego, USA) for use in in vitro PBMC stimulation assays, and GS-9620 was a gift from Gilead Sciences. All of them were used at concentration recommended by manufacturer for optimal in vitro stimulation. Recombinant IFN-α-2a and IFN-λ3 were obtained from PBL. Anti-Human Interferon Lambda Receptor 1 (IFNLR), clone MMHLR-1, neutralizing (MAb) was from PBL; Anti-IFN-α/β Receptor Chain 2 Antibody, clone MMHAR-2, MAB1155 was from EMD Millipore.

Preparation of HBV. Two HepG2-derived cell lines were used for HBV production and purification: the 2.2.15 cell line and AD38 cell line. Infectious particles (Dane particles) were purified by 6% PEG-precipitation and centrifugation from collected cell-free supernatants.

HBV infection of HepG2-NTCP cells and PHHs. HepG2-NTCP cells were infected with HepG2.2.15-derived HBV (2000 viral genome equivalents per cell) overnight in the presence of 4% PEG8000 and 2.5% DMSO. Then, HepG2-NTCP cells were washed 3 times with PBS and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2.5% DMSO. PHHs were infected with HepAD38-derived HBV (500 viral genome equivalents per cell) overnight in the presence of 4% PEG8000. Then, PHHs were washed 3 times with Williams E Medium (Thermo Fisher Scientific) and maintained in Williams E medium supplemented with the Primary Hepatocyte Maintenance Supplement kit (Gibco) and 2% DMSO.

Detection of HBsAg and HBeAg secretion by ELISA. Cell-free supernatants from HBV-infected HepG2-NTCP cells or PHHs were collected and centrifuged at 300 × *g* for 5 min to remove cellular debris, transferred into clean tubes and stored at − 80 °C until antigen measurement. The titers of HBsAg and HBeAg were measured using a commercial ELISA kit (Bioneovan, Beijing, China) according to the manufacturer's instructions.

In vitro GEN2.2 and PBMC stimulation. To determine cytokine production, PBMCs (3×10^6 cells/ml) or GEN2.2 (1×10^6 cells/ml) were stimulated with CpG-A (4 µg/ml), CpG-B (4 µg/ml), CL413 (4 µg/ml), CL267 (4 µg/ml), R848 (4 µg/ml), and GS-9620 (50 nM or 10 µM) overnight.

Total HBV DNA and cccDNA quantification. Total cellular DNA was isolated from HBV-infected PHHs with the NucleoSpin Tissue Kit (Macherey–Nagel). The total HBV DNA level was determined by quantitative PCR (qPCR) using primers specific for HBV DNA: HBV-F, 5′-AGAGGACTCTTGGACTCTCTGC-3′; HBV-R, 5′-CTCCCAGTCTTTAAACAAACAGTC-3′; and the probe pHBV, 5′-[FAM]TCAACGACCGAC CTT[BHQ1]-3′. qPCR was performed with gb Elite PCR Master Mix (Generi Biotech) and TaqMan probe. The level of HBV DNA was normalized to albumin (Alb-F, 5′-GCTGTCATCTCTTGTGGGCTGT-3′; Alb-R, 5′-AAACTCATGGGAGCTGCTGGTT-3′; and Alb-probe, 5′-[FAM]GGAGAGATTTGTGTGGGCATGACA GG[BHQ1]-3′). cccDNA quantification was performed as previously described[50]. Briefly, 1 µg of DNA was treated with 10 units of T5 exonuclease for 2 h. Then, DNA was purified using a DNA Clean and Concentrator Kit (Zymo Research). qPCR was performed with gb Elite PCR Master Mix (Generi Biotech) and specific cccDNA primers and probe. The level of cccDNA was normalized to mitochondrial-encoded cytochrome-c oxidase subunit II (MT-CO2) expression in samples without T5 exonuclease digestion. cccDNA-specific primers and MC-CO2-specific primers were used as previously described[24]. ddPCR was performed using a QX200 Digital PCR Generator and QX200 Droplet Reader (both Biorad) with cccDNA-specific primers and probe. T5 treatment and cccDNA primer specificity was confirmed by comparing the T5-treated and non-treated samples and by comparing cccDNA specific primers with total HBV DNA primers (non-specific cccDNA primers).

Blockade of IFNAR and IFNLAR. IFNAR was blocked by Anti-IFN-α/β Receptor Chain 2 Antibody, clone MMHAR-2, MAB1155 EMD (Millipore) at 5 µg/ml. IFN-λ receptor 1 was blocked by Anti-Human Interferon Lambda Receptor 1, clone MMHLR-1 (PBL) at 5 µg/ml. Mouse IgG1 control from murine myeloma clone MOPC 21 and mouse IgG2a isotype control from murine myeloma clone UPC-10 were used as isotype controls.

Determination of secreted cytokines and chemokines. The quantities of total IFN-α, IFN-γ, IFN-λ1, TNF-α, IL-6, IL-12, IL-8 and IL-10 produced by PBMCs or GEN2.2 were measured in cell-free supernatants after 16 to 20 h culture using Human ELISA Kits (Mabtech).

Statistical analysis. Quantitative variables are expressed as means ± standard error of the mean (SEM). Non-parametric tests were performed due to the nature of the data. First, Kruskal–Wallis was applied, followed by non-parametric post hoc pairwise multiple comparison Mann–Whitney–Wilcoxon test with *p* value adjustment by Benjamini Hochberg method (BH). In case of data depicted in Fig. 3, Dunn's test was performed also with *p* value adjustment by Benjamini Hochberg method (BH). All tests and pictures were computed and rendered in the R software package, figures were finalized in Adobe Photoshop CS. A p value ≤ 0.05 was considered significant.

Ethics statement. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was performed according to local ethical regulations, following approval by the institutional ethics committee (review board) of the Institute of Experimental Medicine and Thomayer Hospital on March 9, 2016 (docket no. 363116 [G-16–03-02]). All liver tissue donors and PBMC donors provided written informed consent for participation in the study in accordance with institutional and regulatory guidelines.

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Author contributions
V.J., J.H., T.H., P.D. and B.L. designed and performed research and analyzed data; J.F., J.C., L.C., S.D. and T.F.B. provided reagents/materials/analysis; V.J., K.C., I.P., I.H. and J.W. designed research, analyzed data, and wrote the paper. I.H. and J.W. conceived and designed the experiments. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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SUPPLEMENTARY MATERIALS

Toll-like receptor dual-acting agonists are potent inducers of PBMC-produced cytokines that inhibit hepatitis B virus production in primary human hepatocytes

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Supplementary Methods

Determination of NF-κB p65 phosphorylation by dynamic phospho-flow cytometry. PBMCs stimulated for 1 h with TLR agonists were fixed in 4% formaldehyde for 15 min, permeabilized by 90% methanol for 30 min, and incubated with Phospho-NF-κB p65 (Ser536) (93H1) rabbit mAb (Cell Signaling, Danvers, MA). APC-conjugated Goat anti-Rabbit IgG polyclonal antibody (Thermo Fisher Scientific) was used as a secondary antibody. Viable PBMCs were gated according to Live/Dead Zombie Green kit. Samples were analyzed using a BD LSR FORTESSA cytometer (BD Biosciences, San Jose, USA) and data were processed using FLOWJO software (Treestar, San Carlos, USA).

XTT test. Cytotoxic effect was analyzed by XTT colorimetric assay. Briefly, 50 µl of 50:1 mixture of XTT labeling reagent (1 mg/ml; Sigma-Aldrich, Life Science) and PMS electron-coupling reagent (0.383 mg/ml; Sigma-Aldrich, Life Science) was added to the wells with HBV-infected PHH and incubated for 4 h in 37 °C in 5 % CO_2. Formation of orange formazan dye was measured in Victor X3 plate reader.

Supplementary Results

Differential potencies of TLR2/7, TLR7, TLR7/8 and TLR9 agonists to induce repertoire of cytokines secreted by stimulated PBMCs.

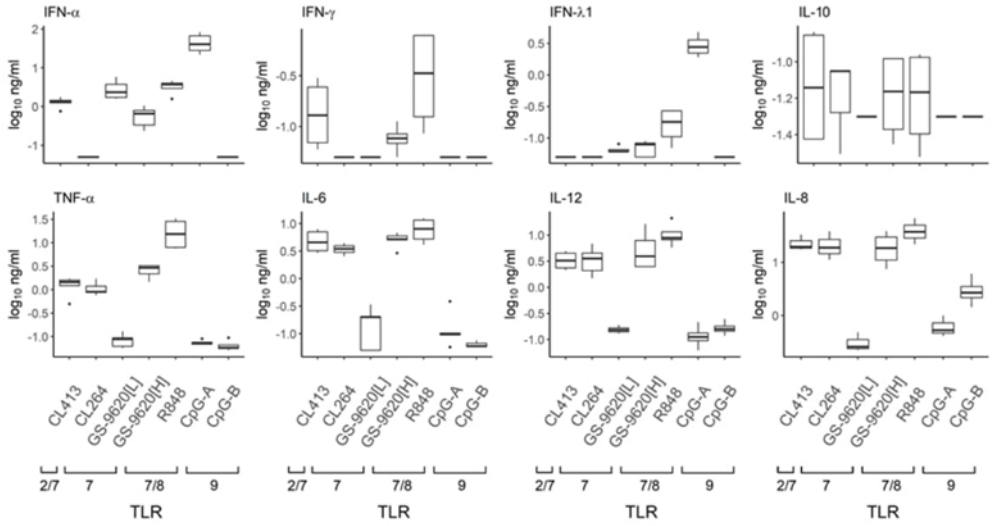


Figure S1. Amounts of cytokines secreted by PBMCs stimulated by different TLR2/7, TLR7, TLR7/8 and TLR9 agonists. PBMCs (N>3) were stimulated with either no agonist, the dual agonist CL413 (5 µg/ml), the TLR7 agonists CL264 (5 µg/ml) or GS9620[L], the TLR7/8 agonists GS9620[H] or R848 (4 µg/ml), or the TLR9 agonists CpG-A (4 µg/ml) or CpG-B (4 µg/ml) for 16 h, and the cytokine levels were determined by ELISA.

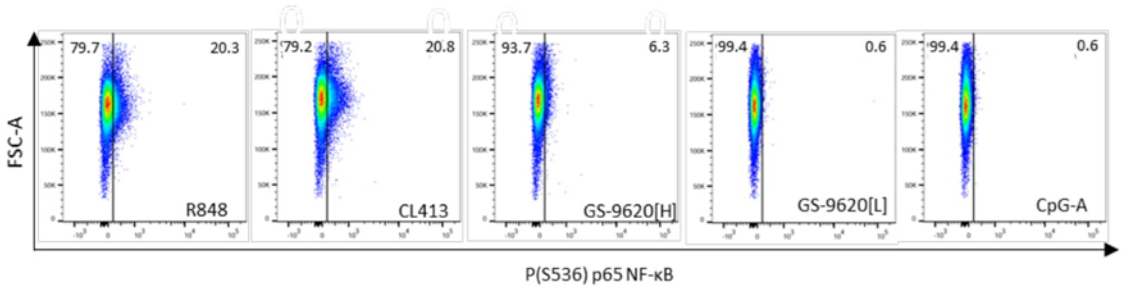


Figure S2. PBMCs were stimulated with the dual-acting agonists R848 (4 µg/ml), CL413 (5 µg/ml) and GS9620[H], or the single-acting agonists of TLR7, GS9620[L], or of TLR9, CpG-A (4 µg/ml), for 1 h, and the expression levels of P(S536) p65 NF-κB cytokine were determined by phospho-flow cytometry. Distribution of phosphorylated p65 NF-κB is shown as a percentage in the upper half of dot-plot diagrams.

Inhibition of HBsAg production from infected PHH exposed to CM from PBMCs stimulated by TLR2/7, TLR7, TLR7/8 and TLR9 agonists

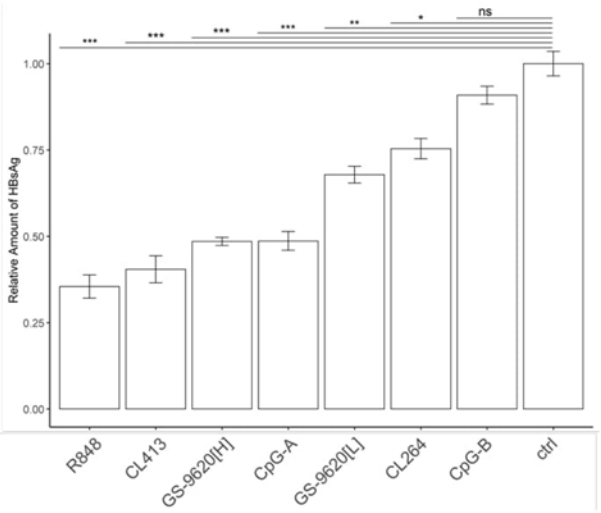


Figure S3. Inhibition of HBsAg production from HBV-infected PHH exposed to PBMC CM. A total of 30,000 PHH were infected with 2000 VGE of HBV and cultured for 3 days before CM (diluted 1:100) from 3×10^6 PBMCs per ml stimulated by agonists of TLR2/7 (CL413), TLR7 (CL264, GS-9620[L]), TLR7/8 (R848, GS-9620[H]), TLR9 (CpG-A, CpG-B) was added. CM was added again 6 DPI. Production of HBeAg was determined by ELISA 9 DPI and normalized to production by HBV-infected PHH in the absence of CM. CM from unstimulated PBMCs was used as a control. Cells were grown for 3 more days. The HBsAg data are shown as mean ± SEM of 5 independent experiments with PHH from three donors. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ pairwise Post hoc Mann-Whitney-Wilcox**on** test. Kruskal-Wallis $p<3.6\times e^{-16}$.

Table S1. Viability of PHH treated with PBMC CM[1)]

TLR agonist	R848	CL413	GS-9620[H]	CpG-A	GS-9620[L]	CL264	CpG-B	Ctrl
Viability (%)	106.4±7.6	104.9±4.1	114.0±4.1	95.5±2,5	97.0±2.4	87.3±1.4	97.2±1.5	100±4.5

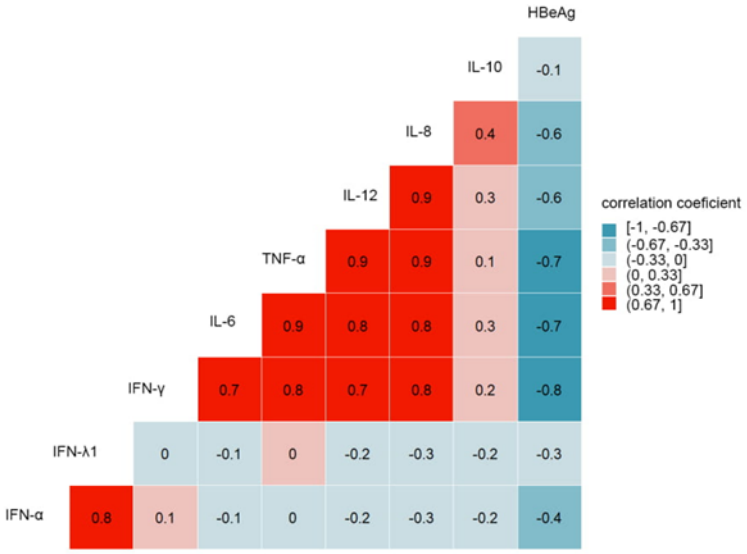
[1)]Determined by XTT test. Conditioned medium (CM) diluted 1:10.

Table S2. Multivariate statistics for HBeAg measurements[1)]

	CL413	CL264	GS-9620[L]	GS-9620[H]	R848	CpG-A	CpG-B
CL413	-	-	-	-	-	-	-
CL264	5.70e-09	-	-	-	-	-	-
GS-9620[L]	5.70e-09	1.40e-07	-	-	-	-	-
GS-9620[H]	2.26e-02	5.70e-09	5.70e-09	-	-	-	-
R848	4.00e-07	5.70e-09	5.70e-09	6.70e-09	-	-	-
CpG-A	1.20e-07	5.70e-09	5.70e-09	2.40e-04	5.70e-09	-	-
CpG-B	9.10e-09	1.50e-08	7.10e-09	9.10e-09	7.10e-09	7.10e-09	-
Ctrl	5.70e-09	5.70e-09	5.70e-09	5.70e-09	5.70e-09	5.70e-09	2.24e-03

[1)]The Kruskal-Walis (p-value <2.2e-16) test indicates differences between groups of agonists. Post hoc Mann-Whitney-Wilcoxon test indicate that all groups of agonists differ.

Table S3. Correlation across PBMC-secreted cytokines[1)]



[1)]The data show correlation coefficients across PBMC-secreted cytokines and HBeAg produced from HBV-infected PHH.

Table S4. Determination of HBV cccDNA by droplet digital PCR

Sample	no T5 treatment		T5 treatment	
	cccDNA primer		cccDNA primer	
	non specific (*copies/µl*)	Specific (*copies/µl*)	non specific (*copies/µl*)	Specific (*copies/µl*)
GS-9620[H]/CM sample 1	7200	86.9	80.8	28.6
GS-9620[H]-CM sample 2	6130	75.5	46.5	26.4
R848-CM sample 1	7290	89.5	105	33.5
R848-CM sample 2	8400	101.5	110	37.4
CL413-CM	6560	78	80.7	32
CTRL-CM	< 10000	121	129	37.3
NO HBV	44.1	No Call	No Call	No Call