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**Dissecting mechanisms underlying increased  
TLR7-mediated IFN $\alpha$  production in pDCs in  
physiological and pathophysiological settings: between  
sex differences and HIV-1-HCV co-infection**

Morgane Griesbeck

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# Thèse de Doctorat de l'Université Pierre et Marie Curie

Ecole doctorale : Physiologie, Physiopathologie et Thérapeutique (ED394)  
Spécialité : Immunologie

Présentée Par

**Morgane GRIESBECK**

Pour Obtenir le grade de

**Docteur de l'Université Pierre et Marie Curie**

Sujet de Thèse

**Dissecting mechanisms underlying increased TLR7-mediated IFN $\alpha$  production in pDCs in physiological and pathophysiological settings:**

**Between sex differences and HIV-1-HCV co-infection**

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Im hokis, gayankis méтч bidi chi mornam jamanagnéré vor miyasin antsousink .Ge chénoragalém kéz vor inzi héd jébdatsir amén ourakh adénéré, yév sirdés jébdétousir téjvar jamanagnéré. Chad ourakh yev hébardém amén inchmov vor miyasin gazmétsink.

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*“Tout est mystère et la clé d’un mystère est un autre mystère”*

Ralph Waldo Emerson



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## List of abbreviations

3C	Chromosome conformation capture
4E-BP	4E binding protein
$\alpha$ -SMA	$\alpha$ -smooth-muscle actin
ADAR-1	Adenosine deaminase acting on RNA-1
Ag	Antigen
AGM	African green monkeys
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase
AMP	Antimicrobial peptide
AP-1	Activator protein 1
AP-3	Adaptor-related protein complex 3
APC	Antigen-presenting cell
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APRI	Aspartate aminotransferase-to-platelet ratio index
Arm	Armstrong strain of LCMV
ART	Antiretroviral therapy
AST	Aspartate aminotransferase
ATF5	Activating transcription factor 5
AZT	Azidothymidine
Bcl2A1	Bcl2-related protein A1
BCR	B cell receptor
BDCA	Blood dendritic cell antigen
Blimp-1	B lymphocyte-induced maturation protein
BLNK	B cell linker
BLOC	Biogenesis of lysosome-related organelle complexes
BM	Bone marrow
BMI	Body mass index
BrdU	Bromodeoxyuridine
BST-2	Bone marrow stromal antigen 2
BTK	Bruton's tyrosine kinase
BTLA	B and T lymphocyte attenuator
CBP	CREB-binding protein
CCL	C-C motif ligand
CCR	C-C chemokine receptor
CD2AP	CD2-associated protein
cDC	Conventional dendritic cells
CDC	Centers for disease controls and prevention
CDLN1	Claudin-1
CEB1	HECT And RLD domain containing E3 ubiquitin protein ligase 5
ChemR23	Chemerin receptor 23
ChIP	Chromatin Immunoprecipitation
Cl13	Clone 13 strain of LCMV
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CMV	Cytomegalovirus
CNS	COP9 signalosome
COP9	Constitutive photomorphogenesis 9
CTCF	CCCTC binding factor
CTL	Cytotoxic CD8+ T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4

CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
DAA	Direct-acting antiviral
Dap12	DNAX activation protein 12
DBD	DNA binding domain
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
Dcp2	mRNA-decapping protein 2
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
Dock2	Dedicator of cytokinesis 2
DR	Death receptor
DS	Degree of skewing
DSIF	DRB sensitivity-inducing factor
dsRNA/DNA	Double-stranded RNA/DNA
DUSP1	Dual specificity phosphatase 1
E1	Estrone
E2	17 $\beta$ -oestradiol
E3	Estriol
EC	Elite controller
ECM	Extracellular matrix
EGF	Epidermal growth factor
EI2AK	Eukaryotic translation initiation factor 2-alpha kinase
eiF	Eukaryotic translation initiation factor
EIF4EBP-1	eIF4E-binding protein 1
EMP	Endothelial microparticule
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERE	Estrogen responsive element
Fc $\gamma$ RII	Fc-gamma receptor II
FISH	Fluorescent <i>in situ</i> hybridization
Flt-3	Fms-related tyrosine kinase 3
Flt-3L	Fms-related tyrosine kinase 3 ligand
FOXO3	Forkhead box protein O3
FPR3	Formyl peptide receptor 3
FSW	Female sex worker
GALT	Gut-associated lymphoid tissue
GAS	Gamma interferon activation site
GAT1	GATA binding protein 1
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPER1	G protein-coupled estrogen receptor 1
GrB	Granzyme B
GWAS	Genome-wide association study
H3K9me2	Histone mark histone H3 lysine 9 dimethylation
HAART	Highly active antiretroviral treatment
HAT	Histone acetyltransferase
HCV	Hepatitis C virus
HCVcc	Cell-culture-adapted strains of HCV
HDAC	Histone deacetylase
HESN	HIV-1-exposed seronegative

HEV	High endothelial venules
HIV-1	Human immunodeficiency virus
HMGB1	High mobility group B1
HSC	Hematopoietic stem cells
HSC	Hepatic stellate cells
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
HS/PC	Hematopoietic stem/progenitor cell
HSV	Herpes simplex virus
I/R	Ischemia/reperfusion
IAD	IRF association domain
ICAM-1	Intracellular adhesion molecule 1
ICOS-L	Inducible T cell co-stimulator ligand
IFIT	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
IFNAR	IFNalpha receptor
IGF1	Insulin-like growth factor 1
IKK	I $\kappa$ B kinase
IL	Interleukin
ILT-7	Immunoglobulin-like transcript 7
Indel	Insertion/deletion
iNKT	Invariant Natural Killer T cell
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IDU	Intravenous drug user
IFI16	Gamma-interferon-inducible protein 16
IP-10	Interferon $\gamma$ -inducible protein 10
IPC	IFN-producing cells
IRAK	IL-1 receptor-associated kinase
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
IRFE	Interferon regulatory factor binding element
ISG	Interferon-stimulated genes
ISGF3	ISG factor 3
ISP	ISG-encoded protein
ISRE	IFN-stimulated response element
ITAM	Immunoreceptor tyrosine-based activation motif
Jak1	Receptor-associated protein tyrosine kinases Janus kinase 1
JFH-1	Japanese fulminant hepatitis-1
JNK	c-Jun N-terminal kinase
KC	Kupffer cells
LAG3	Lymphocyte activation gene 3
LAMP	Lysosomal-associated membrane protein
LAP	LC3-associated phagocytosis
LBD	Ligand-binding domain
LC	Langerhans cells
LC3	Microtubule-associated protein 1A/1B-light chain 3
LCMV	Lymphocytic choriomeningitis virus
LEAP-2	Liver expressed antimicrobial peptide-2
LFA	Lymphocyte function-associated antigen
LILRA4	Leukocyte immunoglobulin-like receptor subfamily A member 4
LNA	Locked nucleic acid
LSEC	Liver sinusoid endothelial cells

LN	Lymph Node
LPS	Lipopolysaccharide
MAKK6	Mitogen-activated protein kinase kinase 6
MAM	Mitochondrial-associated membrane
MAPK	Mitogen-activated protein kinase
MARCH1	Membrane-associated RING finger protein 1
MAVS	Mitochondrial antiviral signaling protein
Mda-5	Melanoma differentiation associated gene 5
MEF	Mouse embryonic fibroblast
MEKK1	Mitogen-activator protein kinase/extracellular signal-regulated kinase (ERK) kinase 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility
MIP	Macrophage inflammatory protein
miR	MicroRNA
MMP	Matrix metalloproteinase
MSM	Men who have sex with men
MT	Microbial translocation
mTOR	Mammalian target of rapamycin
Mx	myxovirus resistance gene
MyD88	Myeloid differentiation primary response gene
NCOR2	Nuclear corepressor 2
NDV	Newcastle disease virus
NASH	Nonalcoholic steatohepatitis
NELF	Negative elongation factor
NES	Nuclear export sequence
NFATc2	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2
NF-κB	Nuclear factor-kappa B
NHP	Non-human primate
NK	Natural killer
NKR-P1A	NK cell surface inhibitory protein P1A
NLRP3	NLR family, pyrin domain containing 3
NLS	Nuclear localization sequence
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitors
OAS	2', 5'-oligoadenylate synthetase
OASL1	2'-5'-oligoadenylate synthase-like protein 1
OCLN	Occludin
ODN	Oligodeoxynucleotide
p70S6K	Ribosomal protein S6 kinase, 70kDa, polypeptide 1
PACSLIN-1	Protein kinase C and casein kinase substrate in neurons 1
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PD-1	Programmed death receptor 1
PDGF	Platelet-derived growth factor
PD-L1	Programmed death ligand 1
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T)-rich
pegIFN	Pegylated interferon
PHH	Primary hepatocyte
PI	Protease inhibitor

PI3K	Phosphoinositide 3-kinase
PIAS1	Protein inhibitor of activated STAT1
PKC	Protein kinase C
PKR	Protein kinase, IFN-inducible dsRNA dependent
PLSCR1	Phospholipid scramblase 1
PLT	Platelet
PP2A	Protein phosphatase 2A
PrEP	Pre-exposure prophylaxis
PRMT1	Protein arginine methyl-transferase 1
PRR	Pattern recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
RAG	Recombination activation gene
RAGE	Receptor for advanced glycation end-products
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBV	Ribavirin
RIG-I	Retinoic Acid Inducible Gene I
RLR	RIG-I-like receptor
RM	Rhesus macaques
ROS	Reactive oxygen species
RPS28	Ribosomal protein S28
Runx2	Runt-related transcription factor 2
SAMHD1	SAM domain and HD domain 1
SCHL11	Schlafen 11
SETB1	SET domain bifurcated 1
SHP-1	SH2 domain containing tyrosine phosphatase 1
Siglec-H	Sialic-acid-binding immunoglobulin-like lectin
SIN3A	SIN3 transcription regulator homologue A
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
Slc15A4	Solute Carrier Family 15 (Oligopeptide Transporter) Member 4
SLE	Systemic lupus erythematosus
SM	Sooty mangabeys
SMART	Strategies for management of anti-retroviral therapy
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
Sp1	Specificity protein 1
SRC-1	Steroid receptor coactivator 1
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
STI	Structured treatment interruption
STI	Sexually transmitted infection
STING	Stimulator of interferon genes
sTNFR	Soluble TNF receptor
SUMO	Small ubiquitin-related modifier
SVR	Sustained virological response
Syk	Spleen tyrosine kinase
TAB2	TAK1-binding protein 2
TAK1	Transforming growth factor- $\beta$ -activated protein kinase 1
TBK1	TANK binding kinase 1
TCR	T cell receptor
Tdt	Deoxynucleotidyl transferase

TE	Transient elastography
TGFβ	Transforming growth factor beta
Tim3	T cell immunoglobulin mucin family member 3
TIMP	Tissue inhibitors of metalloproteinase
TIR	Toll/IL1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAF	Tumor necrosis factor-associated factor
TRAIL	Tumor necrosis factor apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing IFNβ
Treg	Regulatory T cells
TREM	Triggering receptor expressed on myeloid cells
TREX1	Three prime repair exonuclease 1
TRIM	Tripartite motif-containing protein
Tyk2	Tyrosine kinase 2
UBE2N	Ubiquitin-conjugating enzyme E2N
UBE2V1	Ubiquitin-conjugating enzyme E2 variant 1
UNC93B1	Unc93 ( <i>C. Elegans</i> ) Homolog B
USA	United states of America
USP18	Ubiquitin-specific peptidase
UTR	Untranslated region
VCAM-1	Vascular cell adhesion molecule-1
Viperin	Virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible
VL	Viral load
VSV	Vesicular stomatitis virus
WISH	Women's interagency HIV study
XBP-1	X-box binding protein 1
XCI	X-chromosome inactivation

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## Résumé de la thèse

Les interférons de type I (IFN-I) peuvent être produits par toutes les cellules mais les rares cellules dendritiques plasmacytoïdes en sont les principales cellules productrices. Ils induisent de nombreux effets antiviraux et immuno-modulateurs. Un nombre croissant d'études rapporte leur rôle délétère dans certaines maladies auto-immunes et dans différentes infections virales. L'IFN $\alpha$  orchestre de nombreux mécanismes pathogéniques dans l'infection par le virus de l'immunodéficience humaine de type 1 (VIH-1). Bien que les traitements antirétroviraux ont largement contribué à l'amélioration de la qualité de vie des individus infectés par le VIH-1, ces derniers sont enclins au développement de maladies non-liées au SIDA telles que les maladies cardiovasculaires ou hépatiques. Les défauts d'activation immunitaire et d'inflammation persistant chez ces patients et particulièrement ceux liés au dysfonctionnement de la voie de l'IFN $\alpha$  pourraient être en cause. En l'absence d'une cure fonctionnelle, les stratégies visant à décroître les niveaux d'activation immunitaire et d'inflammation, notamment en ciblant la signalisation de l'IFN $\alpha$  sont pertinentes. Cependant, les mécanismes moléculaires associés à un effet immuno-modulateur donné ne sont qu'incomplètement compris. L'étude de modèles physiologiques et pathophysiologiques peut fournir des informations cruciales sur les moyens d'exploiter la signalisation de l'IFN $\alpha$  dans un but thérapeutique.

Les objectifs de ma thèse se déclinent en deux axes :

### **1. Identification des mécanismes responsables de la production d'IFN $\alpha$ plus importante chez les femmes par rapport aux hommes : Contribution du facteur régulateur interféron 5 (IRF5)**

Les pDCs de sujets sains féminins produisent plus d'IFN $\alpha$  en réponse à la stimulation du récepteur Toll-like 7 (TLR7) que les pDCs de sujets sains masculins. Les mécanismes impliqués dans cette différence n'ont été que partiellement identifiés. Des études précédentes ont révélé l'implication de la voie de signalisation du récepteur à l'œstrogène alpha (ER $\alpha$ ). La voie de signalisation de TLR7 des pDCs s'organise autour de la famille des facteurs régulateurs interféron (IRFs). En particulier, IRF7 est connu pour son rôle essentiel dans l'induction de la transcription de l'IFN $\alpha$ . Des parallèles pathophysiologiques avec le lupus systémique érythémateux (SLE), une maladie auto-immune dont la prévalence est plus importante chez les femmes et associée à une activation de la voie de l'IFN $\alpha$ , nous ont conduit à étudier le rôle d'IRF5. En effet, des polymorphismes du gène IRF5, reliés à une expression d'IRF5 et une activation de la voie de l'IFN $\alpha$  accrues, sont associés au SLE. Nos résultats montrent que les pDCs de sujets sains féminins expriment plus d'IRF5 que les pDCs de sujets sains masculins, alors qu'il n'y a pas de différence d'expression d'IRF7. En outre, l'augmentation de l'expression d'IRF5, naturelle ou induite expérimentalement, est associée à une production plus forte d'IFN $\alpha$ . Finalement, nous montrons que, par rapport à des pDCs de souris sauvages, les pDCs de souris dont l'expression du gène *ESR1* (codant pour ER $\alpha$ ) est

invalidée spécifiquement dans les cellules dendritiques ou dans le compartiment hématopoïétique expriment significativement moins d'IRF5 et montrent par ailleurs un défaut de production d'IFN $\alpha$  en réponse à TLR7. Nous démontrons ainsi un mécanisme par lequel la plus forte expression d'IRF5 dans les pDCs chez les sujets sains féminins, sous le contrôle d'ER $\alpha$ , participe à leur production plus élevée d'IFN $\alpha$  en réponse à TLR7.

## **2. Comparaison de la voie de l'IFN $\alpha$ /TLR7 dans les pDCs et des niveaux d'activation immunitaire et d'inflammation chez des patients co-infectés par le VIH-1 et le virus de l'hépatite C et des patients mono-infectés par le VIH-1 sous traitement antirétroviral**

La co-infection par le virus de l'hépatite C (VHC) est aujourd'hui l'une des principales causes de mortalité parmi les individus infectés par le VIH-1. Bien qu'il n'y ait pas de consensus, différentes études ont rapportés des niveaux accrus d'activation immunitaire et d'inflammation chez les individus co-infectés par le VIH-1 et le VHC par rapport aux individus mono-infectés par le VIH-1. L'inflammation est un des facteurs contribuant à la fibrose hépatique. Tant le VIH-1 que le VHC activent et altèrent la voie de signalisation des IFN de type I et de TLR7 associée aux pDCs. La contribution de ces divers effets sur la réponse finale des pDCs reste à déterminer. Nous avons émis l'hypothèse que l'activation immunitaire chronique plus élevée observée chez des individus co-infectés par le VIH-1 et VHC pourrait être due à un dysfonctionnement de la voie de TLR7/IFN-I dans les pDCs. Nous n'avons pas observé de différence d'activation des pDCs et cellules T mais un plus fort épuisement de ces cellules chez les individus co-infectés à fibrose minime ou modéré que mono-infectés. À l'opposé, les patients co-infectés ont une plus d'inflammation. La voie de TLR7/IFN-I dans les pDCs reste plus activée chez les patients infectés par le VIH-1 sous traitement antirétroviral suppressif par rapport aux donneurs sains, comme déterminée par les niveaux d'IRF7 et de STAT1 phosphorylés. Enfin, l'analyse du transcriptome des pDCs révèle une signature de gènes stimulés par l'IFN plus forte chez les patients co-infectés que mono-infectés, qui est associée à la sévérité de l'infection. Les traitements anti-VHC sont aujourd'hui recommandés uniquement pour les patients (co-infectés ou mono-infectés par le VHC) dont le stade de fibrose est relevé. Nos données suggèrent que les patients co-infectés par le VIH-1 et le VHC, même à fibrose minime, pourraient bénéficier d'un traitement plus précoce.

Ce travail de thèse met donc en lumière des mécanismes associés à une plus grande production d'IFN $\alpha$  associée à une inflammation et/ou activation immunitaire délétères et identifie de nouvelles cibles thérapeutiques pour la moduler la voie de l'IFN $\alpha$ .

## Preface

One can say that sex differences and co-infection with the human immunodeficiency virus type 1 (HIV-1) and the hepatitis C virus (HCV) is a surprising association and one would be right on many aspects. However, taking closer look shows otherwise.

Type I IFNs are fascinating. Discovered in 1957, more than half a century ago, for their antiviral properties, there is still plenty we do not know about them. They can be produced by virtually any cell type, but a rare population of immune cells, known as plasmacytoid dendritic cells (pDCs), are recognized as the professional producers of type I IFNs. The identification of the various immunomodulatory functions of type I IFNs mark the cornerstone in our current understanding of their role in immune responses. Their signaling pathways, both upstream and downstream of type I IFN production, could almost be seen as labyrinths given the complexity and the multiple crossroads that can lead to common outcomes. Rather type I IFNs signaling in the current state of knowledge should be considered as a gamebook, in which the narrative branches along various paths, ultimately leading to multiple stories. Indeed, while type I IFNs are the subject of a steadily extensive research, which has uncovered multiple molecules, pathways and effects associated to type I IFNs, one major question remains to be answered. What guide, molecularly speaking, drives a given effect? Or are type I IFNs-driven immune responses inherently indistinctly induced?

Increasing evidences have highlighted a detrimental role of type I IFNs in various viral infections. Notably, pioneering work in HIV-1 infection has highlighted the dichotomous role of IFN $\alpha$ . HIV-1 infection remains a global concern. Although the development of effective antiretroviral treatment has tremendously improved the quality of life in the HIV-1 infected population, they continue to suffer from increased morbidity. In the past decades, the main cause of morbidity has shifted from AIDS-related events to non-AIDS related events including cardiovascular and liver diseases. Importantly, IFN $\alpha$ -driven immune activation and inflammation also contributes to the development of non-AIDS related events. In the absence of functional cure, strategies aimed at decreasing immune activation and inflammation and in particular strategies targeting IFN $\alpha$  signaling are relevant. Physiological and pathophysiological models can provide critical insights on how type I IFNs signaling can be harnessed.



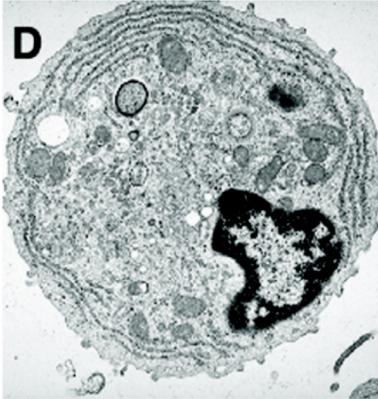
# Introduction



# I. Orchestration of the multifaceted functions of plasmacytoid dendritic cells around IFN $\alpha$ production

## 1. Plasmacytoid dendritic cells: "Professional producers of IFN $\alpha$ "

### 1.1. General characteristics of the plasmacytoid dendritic cells



**Figure 1 : pDC morphology**

Transmission electron microscopy of pDCs shows a nuclei with marginal heterochromatin and a cytoplasm containing well-developed rough endoplasmic reticulum, small Golgi apparatus, and many mitochondria.

From [1]

Plasmacytoid dendritic cells (pDCs) were first referenced as an "enigmatic plasmacytoid T cells" in the paracortical area of reactive lymph nodes in 1958 by Lennert and Remmele. They were then partially characterized in the early 1990s as a rare subset of human peripheral blood leukocytes responsible for high level interferon (IFN) $\alpha$  production but were identified as a subset of dendritic cells (DC) only in the late 1990s. pDCs are relatively quiescent, as suggested by the low rate of incorporation of the DNA base analog bromodeoxyuridine (BrdU) and their turnover is rapid (about 2 weeks)<sup>1,2</sup>. The combination of quiescence and short half-life of pDCs may be a contributing factor towards the small percentage they represent in the immune system<sup>2</sup>. Indeed, pDCs circulate in the peripheral blood of adults and neonates where they represent about 0.1-0.5% of the human peripheral blood mononuclear cells (PBMC)<sup>3</sup>. They reside primarily in the lymphoid organs (lymph nodes (LNs), tonsils, spleen, thymus, bone marrow, and Peyer's patches) in the steady state, entering the LNs from the blood<sup>3</sup>.

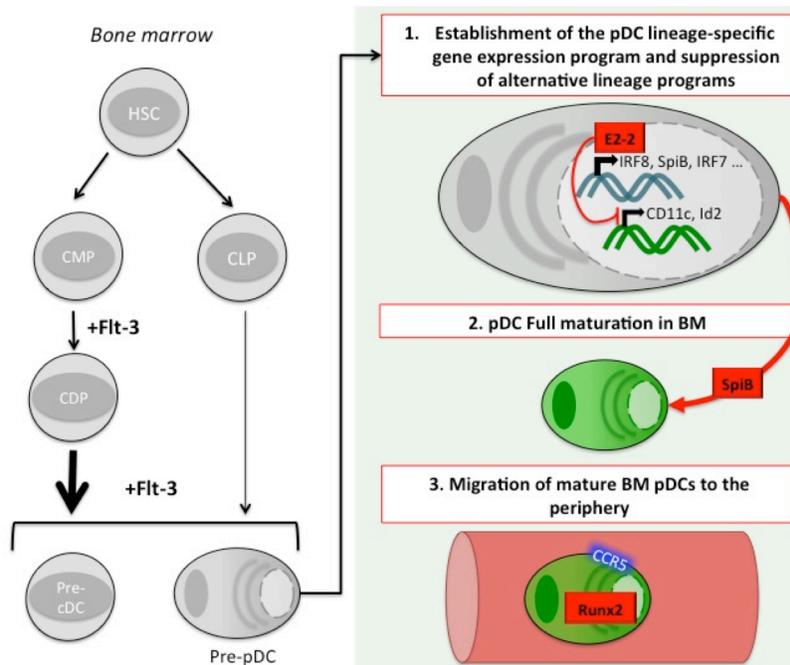
Only small numbers of pDCs are found in peripheral tissues in non-inflammatory conditions with the exception of kidneys, intestine and fetal liver<sup>3</sup>. pDCs are small (8-10 $\mu$ m) and display the morphology of secretory lymphocytes: they are round with a large cytoplasm, have an extensive rough endoplasmic reticulum (ER), and do not exhibit dendrites as found on conventional DCs (cDCs) as shown in Figure 1<sup>1</sup>. They undergo morphological changes upon stimulation with interleukin (IL)-3 and CD40L or with viral stimulation.

pDCs are characterized by the absence of expression of the lineage markers of T lymphocytes (CD3), B lymphocytes (CD19), natural killer (NK) cells (CD56) and monocytes (CD14 and CD16). They express high levels of B cell marker B220/CD45RA, high levels of the alpha chain of the IL-3 receptor (CD123) and low levels of major histocompatibility (MHC) class II molecules. pDCs also express CD4 and several adhesion molecules including CD31, CD43, CD44, CD47, CD62L, CD99, and CD162 (also known as SELPLG, CLA) that may play an important role in the tethering and rolling of pDCs on endothelial cell. Homologs of human pDCs have been identified in mice<sup>4</sup>. pDCs can be identified thanks to the expression of specific lineage markers such as blood dendritic cell antigen

(BDCA)-2 (also known as CD303)<sup>5</sup> and immunoglobulin-like transcript 7 (ILT-7) in humans; or sialic-acid-binding immunoglobulin-like lectin (siglec-H) and bone marrow stromal antigen 2 (BST-2; also known as tetherin) in mice. Nevertheless, those markers need to be used cautiously. BST-2, which is recognized by the antibody mPDCA-1, can only be used to identify murine pDCs in non-inflammatory conditions given its broad inducibility upon activation. BDCA-2 expression is downregulated upon stimulation and its ligation may impair pDC IFN production capacity<sup>5</sup>. Phenotypical and functional differences exist between human and murine pDCs. Human pDCs do not express CD11c contrastingly murine pDCs exhibit low to intermediate levels of CD11c. Conversely murine pDCs do not express CD11b and CD8 $\alpha$ . Murine pDCs from the spleen, but not blood, have been shown to secrete IL-12 upon stimulation, a capacity not shared by human pDC.

pDCs derive from hematopoietic stem cells (HSC) in the bone marrow (BM) as shown in Figure 2. As DCs, they have the unique capability among immune cells to differentiate from both common myeloid progenitors (CMP) and common lymphoid progenitors (CLP)<sup>2</sup>. Nevertheless, the large majority of DCs comes from CMP except in the thymus where at least 50% come from CLPs. pDCs express genes involved in the development of lymphocytes such as terminal deoxynucleotidyl transferase (Tdt), pre-T $\alpha$  or recombination activation gene (RAG) 1/2 but their development is based on the expression of key differentiation factors among which the fms-related tyrosine kinase 3 (Flt-3) ligand (Flt-3L) is of particular importance<sup>2</sup>. Flt-3L and its signaling is crucial for the development pDCs. Consequently, several molecules involved in Flt-3 signaling have been shown to be necessary for pDCs development including signal transducer and activator of transcription (STAT) 3 and STAT5, interferon regulatory factor (IRF)8 (also known as IFN consensus sequence binding protein ICSBP), phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway, and PU.1 which controls in a dose-dependent manner the expression of the Flt-3L receptor. Nevertheless, the molecular pathway transducing Flt-3L signals in pDCs development remain largely unresolved<sup>2</sup>. Once commitment to pDC lineage has occurred, pDCs will only lightly proliferate (if not at all) even upon Flt-3L administration<sup>2</sup>. Interestingly, levels of Flt-3L, which can be secreted by non-hematopoietic tissues and activated T lymphocytes, are increased in inflammatory conditions, suggesting that the pDCs population may differ in inflammatory versus non-inflammatory conditions. The size of the pDCs population is a multigenetic trait. The upregulation of the basic helix-loop-helix transcription factor (E protein) E2-2, otherwise also expressed at significant levels in hematopoietic stem/progenitor cells and B lymphocytes, is crucial for pDCs development and marked the first step of the complex transcriptional program involved in pDC development<sup>2</sup>. The increase in E2-2 concentrations in pDCs is facilitated by the absence of expression of the E protein inhibitor Id2. E2-2 is not only important for the establishment of the pDC lineage-specific gene expression program but also for the suppression of alternative lineage programs. It binds to the promoters of IRF8, SpiB, BDCA-2, ILT-7, leukocyte immunoglobulin-like receptor subfamily A member 4 (LILRA4), Protein kinase C and casein kinase substrate in neurons 1 (PACSIN-1) and IRF7 and repress the expression of CD11c and Id2<sup>2</sup>. The second step of pDCs transcriptional program requires the

expression of SpiB, an Ets-family member controlling expression of the anti-apoptotic gene Bcl2-related protein A1 (Bcl2A1) and responsible for pDCs full maturation in BM. The third and final step involves the pDC-specific marker runt-related transcription factor 2 (Runx2), which is required for the migration of mature BM pDCs to the periphery, in a process that is partially dependent on C-C chemokine receptor (CCR)5<sup>2</sup>. A recent study has questioned the current model conveying that mouse pDCs become fully differentiated in BM and then migrate to the periphery. Indeed, they isolated a subset of circulating CCR9<sup>-</sup> pDC-like precursors from the mouse blood capable to differentiate either to pDCs or cDCs depending on the tissue<sup>6</sup>. As such, although well studied, the pathway of pDC development still requires further scrutiny.



**Figure 2 : pDC development in the bone marrow**

pDCs derive from hematopoietic stem cells (HSC) in the bone marrow (BM). They can differentiate from both common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). Fms-related tyrosine kinase 3 (Flt-3) has a crucial role in this process. Three major steps are involved in pDC development. First, E2-2 promotes the transcription of pDC lineage-specific genes such as IRF8, SpiB and IRF7 while simultaneously suppressing alternative lineage programs, including cDC (CD11c expression). Then, SpiB allows for pDCs full maturation in BM. Finally, migration of mature BM pDCs to the periphery is achieved via Runx2 and is partially dependent on CCR5.

Heterogeneity among pDCs has been poorly characterized but different subsets have been reported. Pelayo et al. reported that two functionally specialized subsets of pDCs arise in BM from progenitors with differential IFN $\alpha$  producing capacities<sup>7</sup>. Similarly, Yang et al. showed that pDC of myeloid versus lymphoid origins have distinct characteristics and function<sup>8</sup>. While challenged by the low frequency of pDCs, the study of pDCs heterogeneity may provide critical information in face of differential functional responses.

## 1.2. Features of a uniquely powerful IFN $\alpha$ secretion capacity

pDCs are recognized as the professional “IFN $\alpha$ -secreting cells”. Several features have been suggested to account, at least partially, for such powerful capacity. As mentioned previously, pDCs exhibit a secretory morphology, which may account for their capacity to produce up to 1000-fold more IFN $\alpha$  than any other cell types. Given that murine cDCs, which lack secretory morphology, are able to produce type I IFN levels comparable to those of pDCs after stimulation through cytoplasmic RNA sensors and that both murine pDCs and cDCs express constitutively the active form of X-box binding protein 1 (XBP-1), the master regulator of secretory function, it seems unlikely that pDC secretory morphology alone is responsible for pDCs capacity to produce high levels of IFN $\alpha$  <sup>2</sup>.

### 1.2.1. Priming of type I IFN signaling via constitutive high level of IRF7

Priming of type I IFNs signaling is a common mechanism in immune cells to ensure the rapid responses to low levels of type I IFNs. Thus, immune cells maintain small amounts of IFN $\beta$  to keep, through an autocrine loop, high levels of two interferon-stimulated genes (ISGs), STAT1 and IRF9, under homeostatic conditions <sup>9</sup>. As discussed later in section II.1.2, STAT1 and IRF9 are two transcription factors involved in the antiviral program induced downstream of interferon alpha receptor (IFNAR) signaling. Similarly, pDCs maintain low levels of IFN $\alpha$  in order to prime their IFN $\alpha$  responses to pathogens. Together with low expression of the translational repressors 4E binding proteins (4E-BPs), this continuous autocrine feedback by low levels of IFN $\alpha$  allows pDCs to maintain constitutively high levels of IRF7<sup>10</sup>. In contrast, in other cell types, the expression of IRF7 is induced upon activation of IFNAR signaling. IRF7 is recognized as the master regulator of type I IFN production <sup>11</sup>. IRF7 has a very short half-life and must therefore be continuously produced. While the baseline IRF7 expression undoubtedly primes pDCs for rapid type I IFN secretion, it seems not to fully account for pDC-specific nature <sup>12</sup>. For example, the PI3K/mTOR signaling pathway, while playing a role on the promotion of anti-inflammatory responses in TLR-stimulated cDCs<sup>2</sup>, promotes the activation and nuclear localization of IRF7 <sup>13</sup>, therefore favoring type I IFNs production in pDCs.

What causes the low levels of basal IFN $\alpha$  in pDCs has not yet been fully elucidated. Spontaneous type I IFN production and pattern recognition receptor (PRR)-triggered IFN production in pDCs do not seem to involve the same signaling pathway. PRR-triggered type I IFN production requires IRF7 but is not significantly affected upon nuclear factor-kappa B (NF- $\kappa$ B) or activator protein 1 (AP-1) inhibition <sup>14</sup>. In contrast, spontaneous type I IFN production requires NF- $\kappa$ B and AP-1 but IRF7 and IRF3 are dispensable <sup>14</sup>. Recent work from Kim and colleagues has shown that spontaneous IFN $\alpha$  production by pDCs depends on cell density<sup>14</sup>. Purified pDCs are known to form large cell clusters upon prolonged culture *in vitro* while *in vivo* pDCs are present as large cell clusters in T cell rich areas of secondary lymphoid organs <sup>1</sup>. Based on the role of receptor activator of nuclear factor kappa-B

ligand (RANKL) in the spontaneous expression of low levels of IFN $\beta$  in osteoclast precursor cells, Kim and colleagues speculated that a growth factor or cytokine-dependent signaling cascade is responsible for NF- $\kappa$ B/AP-1 dependent spontaneous type I IFN production but did not provide any data to support this hypothesis. Interestingly, it has been proposed that commensal microbial flora participate in the basal IFN $\beta$  production in immune cells and therefore in the systemic calibration of type I IFN responses<sup>15,16</sup>. pDCs have been shown to produce IFN $\alpha$  via TLR9 in response to certain non-pathogenic spherical bacteria species including *Lactococcus* and *Streptococcus*<sup>15</sup>. Further investigation of the interactions between commensal microbial flora and pDCs and their potential implications in the priming basal IFN $\alpha$  production is needed.

### 1.2.2. Spatiotemporal regulation of TLR7/9 signaling

Sensing of pathogen by pDCs mainly relies on the Toll-like receptor (TLR)7/9 signaling<sup>17</sup>. TLR9 recognizes unmethylated DNA while TLR7 recognizes single-stranded RNA. Their signaling cascade will be detailed later in section II.2.1. It is generally acknowledged that endosomal trafficking shapes the outcomes of TLR-mediated IFN $\alpha$  responses in pDCs. Indeed, different classes of oligodeoxynucleotides (ODN) have been shown to induce differential IFN $\alpha$  responses via TLR9 in pDCs based on differential endosomal trafficking. CpG-A is a strong IFN $\alpha$  inducer and traffics via early endosome while CpG-B only induces minimal IFN $\alpha$  production and traffics via endolysosomes. Experimental manipulation of CpG-A for delivery to lysosomal compartments leads to a CpG-B-like cytokine response<sup>18</sup>. B cells also express TLR9 but cannot be efficiently activated by CpG-A even if they internalized<sup>19</sup>. Contrarily, CpG-B is a potent B cell mitogen<sup>19</sup>. It is therefore tempting to speculate that cell type differences in TLR7/9 trafficking and processing may account for pDC's unique capacity to produce IFN $\alpha$ .

Proper endosomal trafficking in pDCs, as described in section II.2.2, involves multiple proteins including the solute carrier family 15 (oligopeptide transporter) member 4 (Slc15A4) and the adaptor-related protein complex 3 (AP-3). Both Slc15A4 and AP-3 components are broadly expressed in immune cells, suggesting that their function may involve additional molecular features unique to pDCs. Delivery of TLR7 and TLR9 to the endosomes is dependent on the ER-resident protein Unc93 (*C. Elegans*) homolog B (UNC93B1)<sup>20,21</sup>. It was recently shown that a tyrosine-based sorting motif (Yxx $\Phi$ ) in human UNC93B1 regulates human TLR7, TLR8 and TLR9 in a cell type-, receptor- and ligand-specific manner<sup>22</sup>. Proper TLR7 and TLR9 localization within the endolysosomal compartment is crucial to avoid autoimmune responses. Endosomal TLRs undergo cleavage to gain full functionality<sup>12</sup>. Avalos and colleagues used transgenic TLR9-GFP mice to study cell-specific pattern of TLR9 trafficking in primary antigen-presenting cells (APCs)<sup>23</sup>. They found that proteolysis was a common requirement for TLR9 activation in the primary cell types tested but that the cathepsin requirement, rate of cleavage, and intracellular behavior of TLR9 varied<sup>23</sup>.

Alternatively, Kim and colleagues provided a different interpretation of the spatiotemporal model of pDCs TLR responses, emphasizing a critical role for priming by spontaneous IFN $\alpha$  production. They suggested that the subcellular trafficking of TLR9 signals that induce high IFN $\alpha$  production is related to delayed access to

signaling-competent compartments therefore allowing for pre-activation of the IFNAR-dependent signaling cascade by spontaneous IFN $\alpha$  production and requiring *de novo* protein synthesis<sup>14</sup>. It is known that a first wave of TLR-triggered type I IFNs positively feedbacks in pDCs to amplify the IFN response, notably via activation of IRF8. Such interpretation would consider the spontaneous IFN $\alpha$  production observed specifically in pDCs rather than pDC-specific trafficking pathways as major determinant of pDCs unique capacity to produce high levels of IFN $\alpha$ .

### 1.3. pDC-like models

Three pDC-like cell lines have been described and are increasingly used in the study of pDC functions: the Gen2.2<sup>24</sup>, the CAL-1<sup>25</sup> and the PMDC05<sup>26</sup>. Their characteristics are presented in Table 1. Importantly, cultured PMDC05 cells are a heterogeneous population composed primarily of pDC-like cells (BDCA1<sup>+</sup>CD123<sup>+</sup>) but also of a minor population of cDC-like cells (BDCA1<sup>+</sup>CD123<sup>-</sup>)<sup>26</sup>. These three cell lines have been shown to express TLR7 and TLR9, the main TLRs expressed by primary pDCs<sup>24-26</sup>. However, differential expression of other TLRs has been reported. They all display normal responses to CpG stimulation in terms of both IFN $\alpha$  and tumor necrosis factor (TNF) $\alpha$  production<sup>24-26</sup>. However, TLR7 responses, particularly to synthetic TLR7 ligands, appear rather impaired<sup>24-27</sup>. It was suggested by Pauls et al. that the synthetic TLR7 ligands CL097 and R848 fail to produce significant amounts of IFN $\alpha$  because the activation of I $\kappa$ B kinase (IKK) $\beta$  is not sustained for a sufficient duration<sup>28</sup>. Production of IFN $\alpha$  in Gen2.2 cells therefore depends on the prior production of IFN $\beta$ , which upon secretion into the culture medium activates IFNAR signaling, similarly to what is occurring in all immune cells but in primary pDCs<sup>28</sup>. PDCM05 cells respond to lipopolysaccharide (LPS) stimulation, which is not the case of primary pDCs<sup>29</sup>.

Table 1: Characteristics of pDC-like cell lines

pDC-like cell lines	<b>CAL-1</b>	<b>Gen2.2</b>	<b>PMDC05</b>
Origin	Nagasaki University, Japan	INSERM, Grenoble, France	Niigata University, Japan
Patient	76-year-old male	74-year-old male	67-year-old male
Disease	blastic NK cells lymphomas	pDC-derived acute leukemia	pDC-derived acute leukemia
Phenotype	CD3-CD19-CD14-CD16-CD123+ <b>CD11c+CD56+BDCA2+</b>	CD3-CD19-CD14-CD16-CD123+ <b>CD11c+CD56+BDCA2+</b>	CD3-CD19-CD14-CD16- <b>CD123<math>\pm</math>CD11c<math>\pm</math>CD56+BDCA2-</b>
TLR expression	TLR7/9 <b>TLR2/4</b>	TLR7/9 <b>TLR6-10</b>	TLR7/9 <b>TLR1/2/3/4/8</b>
Cytokines production	IFN $\alpha$ /TNF $\alpha$ upon CpG stimulation IFN $\alpha$ upon FLU stimulation <b>No IFN<math>\alpha</math> upon stimulation with synthetic TLR7 ligands</b>		

Similarly, pDC culture models have been developed in an effort to boost the frustratingly low pDCs numbers. Flt-3L and thrombopoietin (TPO) can synergistically induce the generation of large numbers of pDCs (but also of cDCs and monocytes) from human fetal liver derived or CD34+ cord blood progenitors<sup>30</sup>. Unfortunately, only TLR9-triggered IFN $\alpha$  production was confirmed in these models. pDCs can also be generated (again not specifically) from CD34+ cord blood progenitors after *in vitro* cultivation on the stromal cells OP9 expressing the Notch ligand Delta1 with Flt-3L and IL-7<sup>31</sup>. IFN $\alpha$  production can be detected in this model after stimulation with influenza viruses but not synthetic TLR7 ligand R848<sup>31</sup>. Altogether, these results suggest that pDC-like cell lines and pDC culture models may be useful tools to study some aspects of pDC biology but cannot fully parallel pDC TLR-mediated responses. Primary pDCs therefore remain the only appropriate model to pDCs TLR7-mediated IFN $\alpha$  production.

## **2. pDC functions**

### **2.1 Major role in the orchestration of immune responses following pathogen sensing**

#### **2.1.1. Rapid migration to the sites of inflammation upon pathogen sensing**

Ten TLRs exist in humans and thirteen in mice. All TLRs are membranous except TLR3, 7, 8 and 9 which are endosomal. pDCs selectively express TLR7 and TLR9 in contrast to human cDCs that express TLR1, 2, 3, 4, 5, and 8. TLR expression can be modulated by type I IFNs, TNF and viral infection. pDCs recognize several RNA viruses through TLR7 including influenza virus, the human immunodeficiency virus (HIV-1) and the hepatitis C virus (HCV), leading to subsequent IFN $\alpha$  production<sup>12</sup>. HIV-1 and HCV sensing by pDCs are detailed in sections III.2 and V.2 respectively.

The correct localization of DCs to peripheral tissues and secondary lymphoid organs is a crucial event for optimal immune responses. pDCs rapidly and massively accumulate in inflammatory sites, whether at the viral infections sites, in the context of autoimmune diseases or in response to tissue damage<sup>12</sup>. Transport via the blood stream seems to be the rather exclusive route for pDCs trafficking in both steady-state and inflammatory conditions, and extravasation into LNs through high endothelial venules (HEV) has been postulated to reflect the primary, if not sole, mechanism for entry<sup>32</sup>. In comparison, cDCs have been shown to reach LNs through afferent lymphatic vessels. pDCs engage similar mechanisms as T cells to accomplish LN entry. They rely on L-selectin (CD62L) and P-selectin glycoprotein ligand 1 (PSGL1) in steady state and on E-selectin in inflamed state<sup>32</sup>. Multiple chemotactic signals regulate pDCs trafficking, in contrast to T cells. Both human and murine pDCs upregulate the LN-homing receptor CCR7 upon stimulation with TLR ligands. Conflicting data exist however on the role of CCR7 and its ligand CCL21/CCL19 pathway in resting pDCs. pDCs also express C-X-C chemokine receptor (CXCR)4, the receptor for C-X-C motif ligand (CXCL)12<sup>32</sup>. It has been shown that pDCs purified from chronically HCV infected patients, or exposed *in vitro* to IFN $\alpha$ , acquire the ability to respond to CCR2, CCR5 and

CXCR3 ligands<sup>32</sup>. The exact contribution or concerted function of either of these chemokine receptors remains controversial and may account for differences between human and murine pDCs as well as condition-specific mechanisms. Importantly, the migratory patterns of pDCs from healthy donors to specific chemokines do not always parallel expression of the relevant chemokine receptor<sup>33</sup>.

In addition to chemokines, adenosine and F2L, two agonists released by damaged tissues at the site of inflammation, have the ability to induce pDCs migration through the engagement of the adenosine receptor A1 and formyl peptide receptor 3 (FPR3) and to simultaneously limit the production of proinflammatory cytokines via A2a receptor upregulation<sup>32</sup>. Direct interactions with endothelial cells during migration or indirect interactions via the secretion of cytokines or endothelial microparticules (EMPs) by endothelial cells may greatly impact the function of migrating immune cells. EMPs released from endothelial damage have been shown to specifically mature pDCs and promote their secretion of inflammatory cytokines such as IL-6 but not IFN $\alpha$ <sup>34</sup>. It has been suggested that EMPs may even initiate pDCs migration<sup>34</sup>. Altogether, these data emphasize the complexity of the trafficking pathways in pDCs migration to the inflamed site.

### **2.1.2. Production of IFN $\alpha$ and other cytokines**

The ability to produce type I IFNs in response to viral exposure is common to almost all cells but the amounts, kinetics, and types depend on the cell type. pDCs are recognized as the “professional type I IFN-producing cells”. Indeed, they can produce more than  $1-2 \times 10^5$  IU/ $10^6$  or the equivalent of 10 pg/cell. This is at least 10-fold more efficient than monocytes. pDCs can produce almost all thirteen IFN $\alpha$  subtypes, IFN $\beta$ , IFN $\omega$ , IFN $\tau$ , IFN $\lambda$ 1-3 but not IFN $\kappa$  or type II IFN (IFN $\gamma$ )<sup>35,36</sup>. Of the thirteen IFN $\alpha$  subtypes, IFN $\alpha$ 1 and 2 are the major subtypes expressed by pDCs<sup>35,36</sup>. Type I IFNs are produced by pDCs in response to a wide range of enveloped viruses, parasites such as *Plasmodium falciparum*, and DNA containing unmethylated CpG sequences, typical of microbial DNA. Upon viral infection, pDCs respond quicker and with larger amounts of cytokines than cDCs. Following activation, pDCs can produce other cytokines, including TNF $\alpha$  and IL-6 and inflammatory chemokines such as CXCL9, CXCL10 (also known as IFN $\gamma$ -inducible protein 10; IP-10), macrophage inflammatory protein (MIP)1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), CXCL8 (IL-8), and CCL5 (RANTES), which mediate some of pDCs immune functions<sup>12</sup>. For example, pDC-derived CCL4 is important for NK cell recruitment<sup>12</sup>.

Depending on the activating stimuli, pDCs can be functionally dichotomous. pDCs can either become IFN-producing cells (IPCs), that minimally upregulate maturation molecules but produce high levels IFN $\alpha$ , or become potent APCs, that do not produce IFN $\alpha$  but rather produce NF- $\kappa$ B-dependent inflammatory cytokines such as TNF $\alpha$ , IL-6, and IL-8, and upregulate co-stimulatory molecules for potent presentation of antigen (Ag) to T cells<sup>10</sup>. Whether pDCs become IPCs or APCs seems related to the endosomal compartments to which the stimulating ligands traffic<sup>10</sup>.

### 2.1.3. Antigen presentation

pDCs can efficiently prime CD4<sup>+</sup> T cell responses in LNs, induce Th1 polarization, prime and cross-prime CD8<sup>+</sup> T cell responses but require to be activated via the TLR pathway<sup>12</sup>. As pDCs differentiate to mature APCs, they lose the capacity to produce type I IFN and upregulate MHC class I and II, LN-homing receptor CCR7, and the T cell costimulatory molecules CD40, CD83, CD80 and CD86. pDCs require osteopontin (Opn) for efficient cross-presentation<sup>37</sup>.

pDCs are generally less efficient at exogenous antigen presentation than cDCs but are able to continuously present endogenous viral antigens in their activated state<sup>38</sup> a capacity that is lost by mature infected cDC. Indeed, pDCs continue to synthesize MHC class II molecules and the MHC class II ubiquitin ligase membrane-associated RING finger protein 1 (MARCH1) long after activation. Sustained MHC class II-peptide complex formation, ubiquitination and turnover render pDCs inefficient in the presentation of exogenous antigens. pDC's decreased efficiency at exogenous antigen presentation can therefore be compensated by the abundance of antigens or by continuous availability of endogenous antigen<sup>38</sup>. pDCs may rely on specific antigen-presentation pathways such as the proteasome-independent, endosomal pathway of viral antigen cross-presentation to cytotoxic CD8<sup>+</sup> T lymphocytes (CTL)<sup>39</sup>. DiPucchio et al. showed that influenza virus stimulation induces routing of MHC-I molecules to the cell surface within 30 minutes of exposure, and cross-presentation of Flu antigen to CD8<sup>+</sup> T cells within 4 hours<sup>39</sup>. pDCs may have therefore evolved a rapid mechanism of viral antigen presentation for activation of memory responses. pDCs can acquire exogenous antigens in the form of soluble proteins or cell-derived material to cross-present to CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>12</sup>. pDCs cannot cross-present antigens unless the soluble protein was complexed with specific antibodies<sup>12</sup>.

Human pDCs express several receptors mediating detection and endocytosis of pathogens that can be processed and presented to T cells, including BDCA-2, DEC-205 or dendritic cell immunoreceptor (DCIR; also known as CLEC4A). Binding to the receptors BDCA2/DCIR/Siglec-H inhibits pDC IFN $\alpha$  production<sup>5</sup>. Although BDCA2 and DCIR are downregulated after TLR activation, DEC-205 expression is induced after TLR activation and continues to function as an antigen-internalization receptor. Besides, the Fc-gamma receptor II (Fc $\gamma$ RII; also known as CD32) expressed by pDCs has been involved in the recognition and internalization of complex chromatin-immunoglobulins, virus and tumor antigens. Similarly, it has been shown that complexes between DNA and the high mobility group B1 (HMGB1) (release from necrotic cells) complex can be recognized by receptor for advanced glycation end-products (RAGE) leading to DNA entry to the endosome and TLR9 activation by pDCs<sup>40,41</sup>.

### 2.1.4. Modulation of the innate and adaptive immune responses

pDC-derived type I IFNs have specific effects on different immune cell types and allow notably the orchestration of both innate and adaptive immune responses. First, pDC-derived type I IFNs promote antigen

presentation by cDCs. Type I IFNs enhance pDC cell-surface expression of MHC molecules and co-stimulatory molecules, promote pDC ability to cross-present antigens during viral infections, and pDC migration to LNs, through the upregulation of chemokine receptor expression, therefore increasing via several mechanisms pDC ability to stimulate T cells. Additionally, it is largely acknowledged that pDC-derived type I IFNs enhance NK cell licensing<sup>2</sup>, increasing both NK cell's cytolytic activity and/or their IFN $\gamma$  production. However, it seems that the inflammatory conditions induced by specific viral infections dictate the degree to which direct or indirect effects of type I IFNs modulate NK cell function and which NK cell function is affected<sup>42</sup>. Type I IFNs are required for the activation and expression of cytolytic effector functions and the production of IFN $\gamma$  by NK cells in both influenza virus and vaccinia virus. By contrast, in MCMV infection, type I IFNs-mediated signaling (through STAT1) is required for NK cell accumulation and cytolytic function but not for IFN $\gamma$  production<sup>42</sup>. As with T cells, the ability of type I IFNs to induce or restrict IFN $\gamma$  production by NK cells is related to differential STAT1 and STAT4 signaling with high levels of STAT1 inhibiting IFN $\gamma$  production by NK cells and high basal levels of STAT4 priming NK cells for IFN $\gamma$  production<sup>42</sup>. NK cells and pDCs have been shown to be in close proximity in the T cells areas of human tonsils and to migrate simultaneously to the site of infection. Both immature and TLR7/9 activated pDCs are protected against NK cell-mediated lysis.

Type I IFNs can also influence both CD4<sup>+</sup> and CD8<sup>+</sup> T cells responses, including survival and proliferation, IFN $\gamma$  production, cytotoxic function and memory formation<sup>42</sup>. Type I IFNs can have both inhibitory and stimulatory effects on T cells. Type I IFN mediated upregulation of the programmed death receptor-ligand 1 (PD-L1; also known as CD274 or B7-H1) on TLR-activated pDC is able to suppress T cell activation by binding to programmed death receptor 1 (PD-1) on T cells<sup>42</sup>. As mentioned above, differential outcomes are controlled by expression levels and activation of STAT molecules downstream of IFNAR<sup>42</sup>. It is likely that both the quantity and the timing of type I IFN delivery may be crucial for the consequent adaptive immune responses to infection, as previously reported<sup>42</sup>. pDCs interact with T lymphocytes, invariant Natural Killer T (iNKT) cells and B lymphocytes in the LN. pDCs exhibit variable effects on naïve T cells depending on activation trigger, the resulting cytokines and expression of surface molecules. In response to CpG or viral stimuli, they mainly induce a Th1 phenotype while a Th2 or Th17 phenotypes are induced upon pDC activation by IL-3 or CD40 and TLR7 ligands. The expression of the inducible T cell co-stimulator ligand (ICOS-L) on CpG/IL-3/CD40 or virus-activated pDC has been shown to induce IL-10 production by naïve CD4<sup>+</sup> T cells/regulatory T cells (Treg)<sup>43</sup>. Type I IFNs also shape B cell responses by promoting B cell activation and antibody responses, including class switching, during viral infection<sup>42</sup>. However, it has been suggested that type I IFNs may only be beneficial for the antibody response early in infection and may affect B cells at specific location rather than systematically. pDC-derived type I IFN induce the up-regulation of TLR7 and activation markers on B cells. In addition, IFN $\alpha$  and IL-6 drive B cell differentiation into plasma cells (in concert with T cells)<sup>44</sup>. pDC-B cell crosstalk can occur through CD40-CD40L interaction<sup>44</sup> or through CD70/CD27 interaction. The latter interaction modulates B cell growth, differentiation, and immunoglobulin secretion. Of note, mature pDCs can be stimulated to produce IFN $\alpha$  by

direct cell-cell contact with activated B cells via LFA-1 or platelet endothelial cell adhesion molecule 1 (PECAM-1; also known as CD31) <sup>45</sup>. Recently, Deal et al. showed that pDC-derived type I IFNs are important for the initiation of both B cell activation and the optimal initiation of mucosal and systemic antigen-specific antibody responses to rotavirus infection in both human and mice in a CD40 ligation independent manner <sup>46</sup>. Altogether, these data show that pDCs have a multifaceted role in the regulation of immune responses, through both direct and indirect (via type I IFNs) effects.

## **2.2. Cytotoxic functions of pDCs**

Cytotoxic function of pDCs occurs through several pathways both indirectly by type I IFN secretion and directly via the granzyme B pathway (GrB). Tumor necrosis factor apoptosis-inducing ligand (TRAIL; also known as TNFSF10) promoter exhibits an IFN-stimulated response element and is induced by type I IFNs from TLR7/9-stimulated pDCs via the IFNAR or the PI3K pathways <sup>24,27,47</sup>. pDCs can therefore mediate cell death of TRAIL-sensitive infected cells and tumor cells expressing death receptors (DR) as has been observed in HIV-1 infected individuals where CD4<sup>+</sup> T cells are killed in a TRAIL-dependent manner <sup>48</sup>. This mechanism may contribute to HIV-1 immunopathogenesis as discussed later in section III.4.2. Additionally, type I IFNs can also induce the expression of apoptosis-inducing ligand CD95 ligand (CD95L; also known as FASL) and induce cell death through this mechanism<sup>42</sup>. The cytotoxic properties of activated pDCs may be upregulated by IL-3 under inflammatory conditions <sup>49</sup>. Human pDC constitutively expresses the serine protease granzyme B (GrB) but not perforin <sup>49</sup>. pDCs have been shown to kill erythroleukemic cell line K562 in a GrB-dependent fashion <sup>49</sup>. Inhibition of caspase signaling abrogated K562 killing, suggesting that pDC-derived GrB is delivered into the cytosol of K562 cells where it initiates caspase-dependent cell death. Interestingly TLR7/9 ligands significantly suppressed GrB expression in pDCs <sup>49</sup>. GrB-mediated killing by pDCs was not recapitulated with primary T cells as targets <sup>50</sup>, instead, pDC-secreted GrB suppresses T cell proliferation. Cytotoxic functions of pDCs have only been recently discovered and further research is needed, notably to understand the relative contribution of the different pathways.

## **2.3. Tolerogenic function of pDCs**

According to the activation signals provided by the local environment, activated pDCs display immunogenic or tolerogenic capacities. pDCs contribute to peripheral T cell tolerance in transplantation, tumor escape, oral tolerance, and mucosal tolerance<sup>51</sup>. Despite their reported role in the pathogenesis of certain autoimmune diseases, pDCs may contribute to tolerance to self antigens and regulate the severity of autoimmune diseases<sup>52</sup>. Nevertheless, pDC's tolerogenic function may not be universal but rather be organ-dependent as exemplified by the role of liver pDCs in oral tolerance induction. pDCs tolerogenic function mainly

rely on their ability to induce T cell differentiation into regulatory T cells (Tregs)<sup>52</sup>. They have been shown to preferentially induce IL-10-producing Tregs as compared to transforming growth factor beta (TGF $\beta$ )-producing Tregs, which may be related to ICOS-L upregulation<sup>52</sup>. Additionally, a subset of pDCs expressing lymphocyte activation gene 3 (LAG3) negatively regulates T cell activation and positively regulates Treg function by production of IL-6<sup>53,54</sup>. Importantly, tolerogenic pDC functions were demonstrated to be dependent on indoleamine-pyrrole 2,3-dioxygenase (IDO) in several models, independently of the inflammatory environment<sup>52</sup>. pDCs therefore demonstrate both inflammatory and suppressive properties adding to the complexities they exert on modulating the larger immune response.

Among the several functions of pDC, type I IFN production has a multitude of effects including the mentioned modulation of innate and adaptive immune response and induction of cellular cytotoxicity. Given their importance, the production and activity of type I IFN needs to be finely tuned. Figure 3 summarizes the multiple layers of regulation that are in place at the molecular level to control type I IFN.

## II. Regulation of the TLR7/IFN pathway in pDCs

### 1. Fine tuning of type I IFN responses

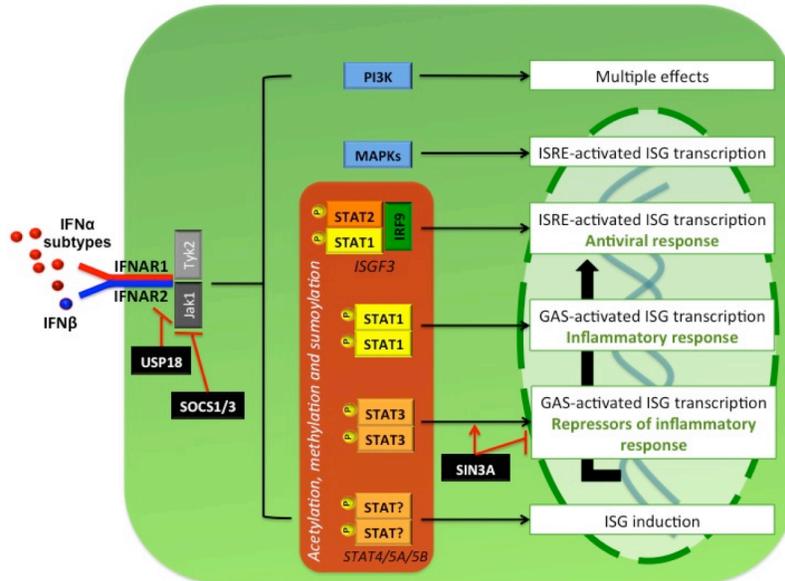
#### 1.1. The type I IFNs family and the IFN $\alpha$ subtypes: Between redundancy and specificities

The type I IFN family is a multi-gene cytokine family that encodes 13 subtypes of IFN $\alpha$ , a single IFN $\beta$  protein, and the poorly defined IFNs  $\omega$ ,  $\epsilon$ ,  $\delta$ ,  $\zeta$  and  $\tau$ <sup>42</sup>. The protein sequence of all the different IFN $\alpha$  subtypes is highly conserved with 75-99% amino acid sequence identity and the length of the protein is about 161-167 amino acids. The IFN $\alpha$  subtypes share about 35% identity with IFN $\beta$ . Type I IFNs can be induced by the stimulation of various PRR. In most cases, IRF3 and IRF7 are the fundamental IRFs that are required for type I IFNs transcription. Others IRFs, including IRF1, IRF5 and IRF8 are also involved in type I IFNs induction. Usually, *IFNB* and *IFNA4* genes are induced in the initial wave of transcription that relies on IRF3, as IRF7 is not constitutively expressed (except in pDCs). This initial IFN burst triggers the transcription of IRF7, which then mediates a positive feedback loop, leading to the induction of a second wave of gene transcription, including additional IFN $\alpha$ -encoded genes<sup>55</sup>. Differences in the expression levels and post-translational modifications of IRFs may contribute to differential induction of IFN $\alpha$  subtypes<sup>55</sup>. Of note, both IRF5<sup>56</sup> and IRF7<sup>57</sup> have splice variants, which could differentially regulate IFN $\alpha$  subtypes expression.

All type I IFNs signal through the same receptor, IFNAR. IFNAR is a heterodimeric transmembrane receptor composed of IFNAR1 and IFNAR2 subunits<sup>42</sup>. IFN $\alpha$  subtypes bind IFNAR with close range of affinity: IFNAR1 at  $\mu$ M affinity and the IFNAR2 at nM affinity<sup>58</sup> with IFN $\alpha$ 1, IFN $\alpha$ 10 and IFN $\alpha$ 17 having lower binding affinities<sup>58,59</sup>. However, the density of the receptor on the surface of a specific target cell affects the biological activities of the subtypes<sup>60</sup>. Moraga et al. showed that IFN $\beta$  was more potent than IFN $\alpha$ 2 in anti-proliferative activity in cells with scarce receptors, while the opposite trend was observed in cells with abundant receptors<sup>60</sup>. Interestingly, the affinities were clearly associated with the anti-proliferative potency of the different IFN $\alpha$  subtypes, but no correlation with the antiviral activities of the subtypes has been found, as determined in vesicular stomatitis virus (VSV) and encephalomyocarditis virus neutralization assays<sup>58</sup>. Understandably, cells need to be able to sense very low levels of type I IFNs and act very fast in order to clear viral infections in their initial stages. In contrast, anti-proliferative activity, which is often linked with apoptosis and tissue damage, needs to be under tighter control to prevent unnecessary damage. It has been previously shown that type I IFN anti-proliferative activity benefits from an increased binding affinity<sup>61</sup>. It seems therefore biologically relevant that the anti-proliferative activities of IFN $\alpha$  subtypes are more sensitive to variations in the receptor affinities among IFN $\alpha$  subtypes than antiviral activities<sup>62</sup>. Upon ligand binding, IFNAR1 undergoes conformational change to allow the formation of a ternary complex that is stable enough to facilitate phosphorylation *in trans* between receptor-associated protein tyrosine kinases Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). The results presented by Lavoie et al. therefore also suggests that antiviral potency of the IFN $\alpha$  subtypes is dictated by

stability of the ternary complex and not its rate of formation. Moreover, phosphorylation of STAT1 and STAT3 have been shown to be differentially induced by IFN $\alpha$  subtypes. IFN $\alpha$ 1 but not IFN $\alpha$ 2, 4 and 5 lead to the phosphorylation of STAT3 while all induce STAT1 phosphorylation<sup>63</sup>. Differences in the downstream signaling cascades, notably in STATs phosphorylation, may alter the transcription of ISGs as discussed below.

A limited number of studies using virus infection models have evaluated the distinct anti-viral activities of specific IFN $\alpha$  subtypes. But it seems that both the IFN-producing cell type and the type of infecting virus differentially affect the expression and action of different IFN $\alpha$  subtypes<sup>64</sup>. pDCs can produce high levels of all type I IFNs at the exception of IFN $\alpha$ 6 and IFN $\alpha$ 8 which are only weakly induced in pDCs<sup>64</sup> and IFN $\alpha$ 7 which is not induced by TLR ligand, imiquimod,-stimulated pDCs<sup>12</sup>. IFN $\alpha$ 5 has been shown to be the main subtype produced in the liver<sup>64</sup> and is inducing the highest 2'-5'-OAS expression in hepatocytic cells, most likely in response to its stronger activation of STAT1 and Tyk2 signaling compared to IFN $\alpha$ 2. Interestingly, IFN $\alpha$ 8 is the most effective IFN $\alpha$  subtypes in suppressing HCV replication while IFN $\alpha$ 1 was the least effective<sup>64</sup>. In addition, IFN $\alpha$ 1/13 is the weakest inducers of ISGs compared to all other IFN $\alpha$  subtypes in CpG and imiquimod stimulated-PBMCs. IFN $\alpha$ 14, 10, 2, 6, 7 and 8 are in contrast very potent inducers of interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), including CXCL10, CXCL11, ISG15 and CCL8<sup>64</sup>. Variable effects on T cell and DC function and B cell proliferation have also been reported for IFN $\alpha$  subtypes. But most of the studies have focused on the impact on viral titers and did not investigate immunomodulatory effect of IFN $\alpha$  subtypes. Nevertheless, it has been shown that IFN $\alpha$ 11 is specifically required for optimal NK cell responses<sup>64</sup>. In addition, the variable effect of IFN $\alpha$  subtypes on specific cell types may be linked to the activation of alternative receptors. IFN $\alpha$  has been shown to induce variable signaling through the complement receptor type 2 (CR2/CD21) in B cells<sup>65</sup>. Taken together, these data suggest that individual IFN $\alpha$  subtypes may induce the expression of a specific pattern of ISGs owing to both different receptor affinities as well as cell type-specific responses. The maintenance of numerous IFN $\alpha$  subtypes throughout evolutionary history must be a survival strategy. One can imagine that the numerous IFN $\alpha$  subtypes form an arsenal of responses against different viruses with specific type I IFN subtype capable of inducing subsets of ISGs that are more effective at inhibiting different viruses<sup>66</sup>. However, until now, no unique function has been attributed to any given subtype<sup>58</sup> and the redundancy of the IFN $\alpha$  subtypes requires further investigation.



**Figure 3: Diversity of IFNAR signaling**

Activation of IFNAR by type I IFNs activate diverse downstream pathways, leading to diverse biological effects. Binding to IFNAR by IFN $\beta$  or a unique IFN $\alpha$  subtype may trigger specific pathways. The canonical STAT1/STAT2/IRF9 signaling complex (also known as ISGF3) binds to ISRE sequence in gene promoters, leading to induction of a large number of ISGs, involved in the antiviral response. Type I IFNs can also signal through STAT1 homodimers, which are more commonly associated with the IFN $\gamma$ -mediated signaling pathway and promote the induction of an inflammatory response. Other STAT heterodimers and homodimers may also be activated downstream, including STAT3, STAT4 and STAT5. SIN3A mediates repression of STAT3-mediated transcription. In addition, post-translational modifications of STATs may alter their transcriptional activity. The MAPKs and PI3K pathways are also activated. Negative regulators of IFNAR signaling include SOCS1/3 and USP18. Adapted from [42]

### 1.2. The canonical type I IFN-induced signaling pathway or Jak-STAT pathway

IFNAR engagement through binding of IFN $\alpha$  or IFN $\beta$  activates Jak1 and Tyk2. The Janus kinase–signal transducer and activator of transcription (Jak-STAT) is used for all IFNs (I, II and III). In the canonical type I IFN signaling pathway, activated Jak1 and Tyk2 phosphorylate STAT1 and STAT2 molecules, leading to their dimerization and nuclear translocation. They form a trimolecular complex involving IRF9 and referred as the ISG factor 3 (ISGF3). ISGF3 recognizes IFN-stimulated response elements (ISREs; consensus sequence TTCNNTTTC) and activates the transcription of ISGs <sup>67</sup>. Importantly, type I IFNs can also signal through STAT1 homodimers, which are more commonly associated with IFN $\gamma$ -mediated signaling, therefore allowing for the recognition of gamma interferon activation site (GAS) elements (GAS; consensus sequence TTCNNGAA) <sup>67</sup>. Type I IFNs can also signal through STAT3, STAT4, STAT5A and STAT5B <sup>67</sup>, therefore further diversifying the signal downstream of IFNAR. The relative expression of STATs is an important determinant of the pattern of IFNAR-induced STAT activation <sup>67</sup>. Indeed, STAT3 acts as a negative regulator of type I IFN-mediated antiviral response by restricting

STAT1-mediated inflammatory signaling downstream of IFNAR and activating genes that suppress inflammatory responses<sup>67</sup>. Furthermore, upon type I IFNs stimulation, STAT3-mediated gene induction is suppressed by the binding of the co-repressor complex SIN3 transcription regulator homologue A (SIN3A) to IFN $\alpha$ -activated STAT3<sup>67</sup>. Transcriptional activity downstream of IFNAR is also controlled by post-translational modification of STATs. For instance, STAT1 transcriptional activity can be enhanced by serine phosphorylation mediated by various members of the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) families<sup>67</sup>. It was recently shown that STAT1 is further phosphorylated in the nucleus by cyclin-dependent kinase 8-dependent after STAT1 binds DNA to enhance the transcription of type I IFNs<sup>64</sup>. The MAPK and PI3K/mTOR pathways can also be activated downstream of IFNAR<sup>42</sup>. They promote the translation of ISGs via the inactivation of the translational suppressor eIF4E-binding protein 1 (EIF4EBP-1) and the activation of the ribosomal protein S6 kinase, 70kDa, polypeptide 1 (p70S6), which in turn activates eIF4 translation initiation factors. STATs can also be modulated via acetylation, methylation and sumoylation<sup>67</sup>. For example, protein inhibitor of activated STAT1 (PIAS1), a small ubiquitin-related modifier (SUMO) ligase, can suppress the binding of STAT1 to DNA<sup>67</sup>. The interactions between STATs and type I IFNs therefore produce a complex series of outcomes that can enhance and suppress immune stimulation.

Cellular responses to IFNAR ligation are modulated in a cell type-and context-dependent manner<sup>68</sup>. During the course of an immune response, the magnitude of the IFNAR response needs to be restricted to avoid toxicity and can be accomplished by the regulation of the expression and post-translational modification of IFNAR and JAK–STAT signaling components. In addition, specific downstream ISG that cooperate with STATs and modulate chromatin states at target gene loci are induced. Regulation of IFNAR signaling also occurs via the regulation of the pattern of protein expression by modifying factors that mediate translation. Cellular responses to IFNAR ligation are therefore also modulated by suppressive mechanisms used by pathogens. The strategies used by HIV-1 and HCV to suppress type I IFNs signaling will be discussed later in the manuscript.

### **1.3. Modulation of type I IFN signaling**

In order to limit cellular IFN responsiveness, cell surface IFNAR expression is downregulated, an evasion mechanism that is commonly used by viruses and tumor cells<sup>67</sup>. Different signaling pathways are involved in IFNAR downregulation, including IL-1, TLRs, immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors signaling pathways and oxidative or metabolic stress. Casein kinase II (CK2)-mediated phosphorylation of IFNAR cytoplasmic domain increases receptor internalization, ubiquitinylation and degradation<sup>67</sup>.

In parallel, negative feedback loops are implemented to limit the extent and duration of type I IFN responses. Different negative regulators are transcriptionally induced upon IFNAR signaling. Two prominent examples are the suppressor of cytokine signaling (SOCS) 1/3, which competes with STATs for binding to IFNAR

and suppresses Jak1 activity, and ubiquitin specific 18 (USP18; also known as UBP43), which displaces Jak1 from IFNAR2. Forkhead box protein O3 (FOXO3) acts as a repressor of type I IFN responses at baseline as well as during the resolution phase of an infection by targeting IRF7<sup>69</sup>. The ISG 2'-5'-oligoadenylate synthase-like protein 1 (OASL1) mediates the suppression of IRF7 translation<sup>67</sup>. miRNAs can also be induced to suppress IFNAR signaling and more generally control TLR signaling<sup>67</sup>. miR-146a, which is induced by type I IFNs and by TLR7/9 agonists in human pDCs<sup>70</sup>, downregulates IRAK1 and TRAF6 expression and regulates STAT1<sup>71</sup>. miR-155 and miR-155\*, which are processed from the same precursor, have opposing roles in controlling TLR-induced IFN production by human pDCs<sup>72</sup> with miR-155\* augmenting type I IFN expression by suppressing the negative TLR7 signaling mediator IRAK1. These various routes of suppressing type I IFN responses can be beneficial in tapering the inflammatory response but can also be exploited in disease settings to decrease the immune activity of type I IFN.

In addition to altering IFNAR access and type I IFN stimulated negative feedback loops, the chromatin state at target gene loci can also modulate the access of STATs to their target genes. The low abundance of CpG islands in ISG promoters predisposes it to high nucleosome occupancy and confers a requirement for nucleosome remodeling<sup>67</sup>. STAT1/2-mediated recruitment of p300, CREB-binding protein (CBP) and GCN5 lead to histone H3 and H4 lysine acetylation in nucleosomes on ISGs promoters and subsequent transcriptional activity<sup>67</sup>. Type I IFNs also induce H2B ubiquitylation, a positive mark that promotes type I IFN-driven ISG transcription and is mediated by the E3 ubiquitin ligase BRE1<sup>73</sup>. Interactions of STATs with co-activators, co-repressors, chromatin-modifying complexes and transcription elongation factors modulate the outcome of IFNAR signaling. For example, negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) proteins restrain the transcription of ISGs after type I IFNs stimulation by promoting the pausing of Pol II<sup>67</sup>. STATs can also bind to distal regulatory elements, such as enhancers. Access to such element is determined during cell differentiation by lineage-determining transcription factors so that the enhancer repertoire is cell type specific<sup>67</sup>. STAT1 primes and/or activates enhancers genome-wide during the differentiation of immune cells, thus reprogramming cellular responses to subsequent stimuli<sup>67</sup>. Histone mark histone H3 lysine 9 dimethylation (H3K9me2) at ISGs attenuates their expression upon type I IFNs stimulation in a cell lineage-specific manner<sup>67</sup>. Epigenetic regulation is therefore an important mechanism to control type I IFNs responses in a cell type-specific manner.

#### **1.4. Stochastic nature of ISGs expression**

Even in populations of nearly identical cells, not all stimulated cells produce type I IFNs and express ISGs<sup>67</sup>. Bimodal variation in mRNA abundance and splicing patterns, while also occur for very highly expressed genes at the population average, is not a universal feature of immune response transcripts. Bimodal activity of regulatory circuits involving STAT2 and IRF7 has been suggested to be responsible for the stochastic nature of

ISGs expression<sup>67</sup>. Biologically, such nature may lead to the presence of both cells massively producing type I IFNs to fight against the infection but prone to cell death, together with preserved 'low IFN producers' to maintain the viability of tissues<sup>67</sup>. Type I IFN signaling is astonishingly diverse and redundant. However, the downstream immunological effect of such tuning mechanisms remains to be determined but would provide crucial information for therapeutic targeting. While most cell types can elicit a type I IFN response by activating the TLR, RLR and Jak-STAT pathways, pDCs are the "professional" type I IFN-producing cells. The signaling pathway leading to type I IFN production in pDCs exhibit specific characteristics as schematized in Figure 4.

## 2. Regulation of the TLR7/TLR9 pathway in pDCs

### 2.1. Signaling through endosomal TLR7 and TLR9

TLR7/TLR9 stimulation provide the main basis of upstream pathways to type I IFN production in pDCs. TLR7 recognizes guanosine-uridine-rich single-stranded RNA (ssRNA) as well as the potent synthetic antiviral imidazo-quinolines. CpG RNA motifs in a uridine-rich context found in viral RNA can also contribute to RNA recognition via TLR7<sup>12</sup>. Recognition by TLR7 and TLR9 occurs in the endosomal/ lysosomal compartments following acidification and require the cleavage of TLRs by endopeptidase and cathepsins<sup>12</sup>. Delivery of TLR7 and TLR9 to the endosomes is dependent on the ER-resident protein UNC93B. The endosomal and lysosomal localization of TLR7 and TLR9 in activated pDCs is critical. While allowing for recognition of internalized material, it is also prevents inappropriate activation by self-DNA/RNA.

Upon ligand engagement, TLR7/9 recruit the key adaptor molecule myeloid differentiation primary response gene (MyD88) through their Toll/IL1 receptor (TIR) domains, which in turn recruits IL-1 receptor-associated kinase (IRAK) 4. The activated IRAK4 interacts with and phosphorylates IRAK1, then IRAK4/IRAK1 complex dissociates from MyD88 and associates with tumor necrosis factor-associated factor (TRAF) 6 to trigger the downstream signaling pathway<sup>17</sup>. IRAK2 is critical in late-phase TLR responses and sustained proinflammatory cytokine production in pDCs and has recently been implicated in the negative regulation of type I IFN responses in pDCs<sup>17</sup>. While IRF3 expression is sufficient for induction of IFN $\beta$ , IRF5 and/or IRF7 are required for induction of IFN $\alpha$  gene expression. IRF7 is the well-recognized master regulator of type I IFNs. pDCs express constitutively high levels of IRF5 and IRF7 mRNA and IRF7 protein (higher levels than monocytes)<sup>35</sup>. IRFs need to translocate into the nucleus for their transcriptional activity. Nuclear translocation of IRF7 requires its activation through post-translational modifications<sup>11</sup>. IRF7 is recruited by MyD88 complex formed by MyD88, IRAK1, IRAK4, and TRAF6. TRAF6 functions together with the E2 enzyme complex formed by ubiquitin-conjugating enzyme E2N (UBE2N; also known as Ubc13) and ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1; also known as Uev1A) to catalyze the K63-linked polyubiquitination of IRF7, a post-translational modification required for its activation<sup>17</sup>. Viperin, a type I IFN inducible gene residing in the cytoplasmic lipid-

enriched compartments in pDCs, has been shown to interact with IRAK1 and TRAF6 and facilitates the K63-linked ubiquitination of IRAK1, which promotes the nuclear translocation of IRF7<sup>17</sup>. In addition, IRF7 has been shown to interact with TRAF3, I $\kappa$ B and Opn<sup>37,74</sup>. The TANK binding kinase 1 (TBK1), IKK $\alpha$  and IKK $\beta$  are also involved in IRF7 activation<sup>17,28</sup>. NF- $\kappa$ B inducing kinases act as an upstream kinase of IKK $\alpha$  in activation of IRF3/7 and may play important role in TLR7/9-mediated type I IFNs production<sup>17</sup>. But the relative contribution of the different aforementioned kinases in IRF7 phosphorylation remains largely unknown. Adding to the complexity, PI3K/mTOR pathway is also required for IRF7 nuclear translocation<sup>13,75</sup>. Stimulation of TLR7 and TLR9 do not produce the same responses and Bruton's tyrosine kinase (BTK) has been show to regulate TLR9 but not TLR7 signaling in pDCs<sup>76</sup>. In addition to type I IFNs, TRAF6 mediates the activation of transforming growth factor- $\beta$ -activated protein kinase 1 (TAK1) and mitogen-activated protein kinase kinase 6 (MKK6) leading to the activation of NF $\kappa$ B, c-Jun N-terminal kinase (JNK) and MAPKs, leading to the production of proinflammatory cytokines TNF $\alpha$  and IL-6<sup>17</sup>. In human pDCs, crosslinking of CD300a and CD300c inhibits HLA-DR expression and reduces TNF $\alpha$  and IL-6 production while greatly enhances IRF7 expression and IFN $\alpha$  secretion by pDCs<sup>17</sup>. Therefore the upstream signaling of TLR7/9 in pDCs, although can both induce type I IFNs, do not result in the same immune response.

## 2.2. Endosomal trafficking

The analysis of the outcome of TLR9 responses by different class of CpG ODNs has highlighted endosomal trafficking as a critical determinant of the outcome of TLR7/9 responses. The monomeric CpG-B (ODN 2006 or K) initiates the rapid activation of TLR9 and its signaling cascade, with type I IFN production seen as early as 2-4 h after stimulation<sup>14</sup> but IFN $\alpha$  is only induced at low levels by pDCs. CpG-B has been shown to traffic through late endosomes and lysosomes<sup>18</sup>. In contrast, CpG-A (ODN 2216) stimulation results in sustained high IFN $\alpha$  production with minimal upregulation of the T cell costimulatory molecules CD80, CD86 and HLA-DR<sup>12</sup>. CpG-A forms aggregates and are retained in early endosome<sup>18</sup>. Signals emanating from TLR9 are therefore determined by the compartment where the receptor is activated. Prolonged activation of the MyD88/IRF7 signaling complex occurs in the early endosomal compartment, therefore contributing to increased IFN $\alpha$  production<sup>18</sup>. While NF- $\kappa$ B activation proceeds as the default response from the early endosome or early phagosome, the formation of a specialized signaling compartment is required for type I IFN production. The nature of such complex is controversial.

TLR9 signals that induce strong IFN $\alpha$  production involve the trafficking of TLR9 to the acidic lysosomal-associated membrane protein (LAMP) 2+ lysosome-related organelles from which IRF7 signaling is initiated via TRAF3 recruitment. Such trafficking requires the AP-3 complex and the transmembrane protein Slc15a4<sup>77</sup>. It was shown that AP-3 deficient pDCs are unable to produce type I IFN but have intact proinflammatory cytokines production<sup>77</sup>. Robust IRF7 activation can also be triggered LAMP1+ phagolysosome and is associated with

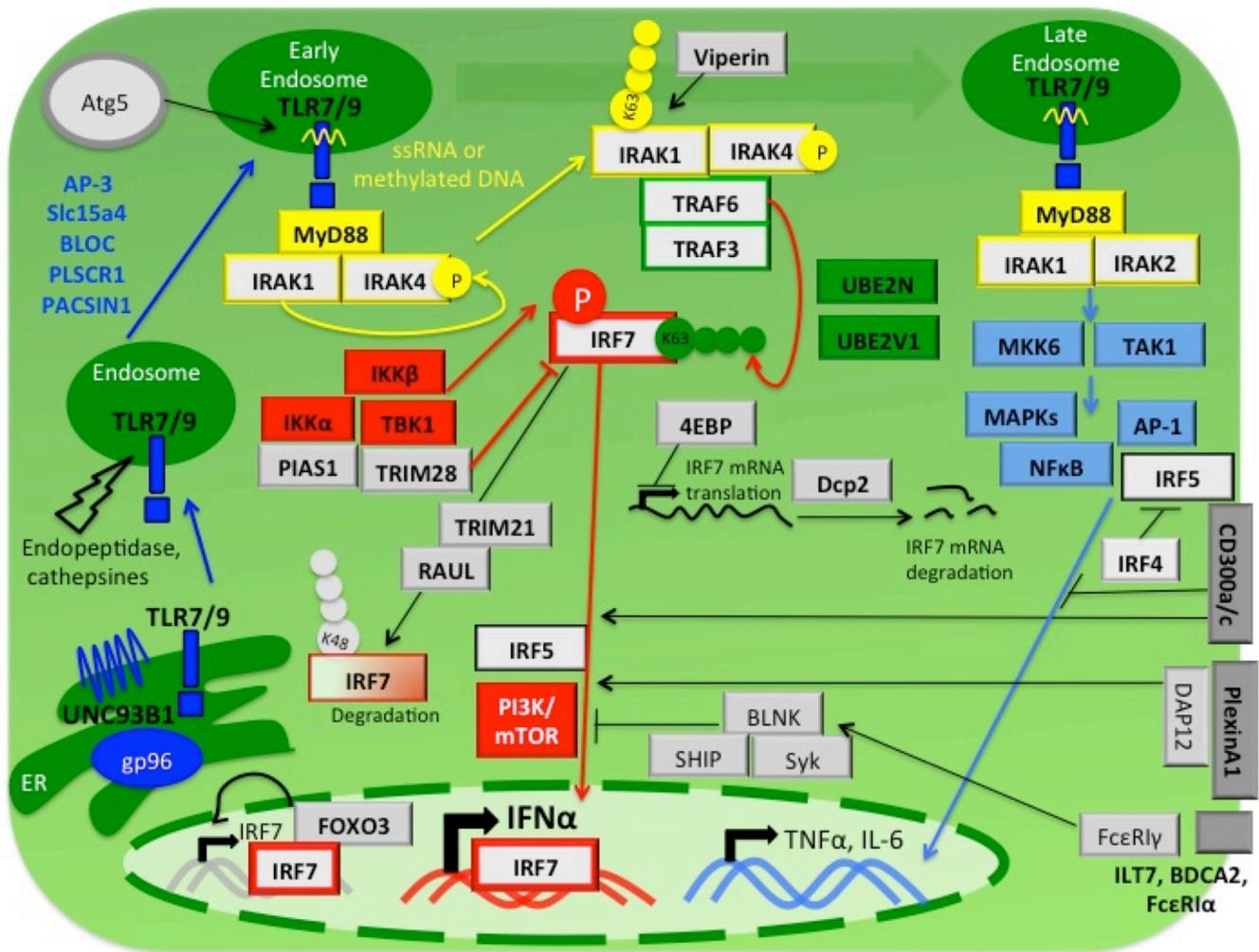
microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP) but independent of AP-3<sup>78</sup>. This mechanism was shown in the context of FcγR-dependent internalization of DNA-containing immune complexes.

Multiple molecules involved in TLR7/9 trafficking and processing have been demonstrated to be required for pDC TLR responses, including the biogenesis of lysosome-related organelle complexes (BLOC)-1, BLOC-2 and BLOC-3 and the solute carrier protein superfamily member Slc15a4 and the phospholipid scramblase 1 (PLSCR1)<sup>17</sup>. Heat shock protein gp96 regulates intracellular trafficking of TLR9 possibly through the proteolytic processing and conformational stability of TLR9<sup>17</sup>. PACSIN-1, which links membrane trafficking with the cytoskeleton and regulates the vesicle trafficking, has been shown to play a specific role in the type I IFN signaling cascade<sup>17</sup>. pDCs constitutively contain autophagosomes which they may use to deliver some ligands to nucleic acid sensors<sup>79</sup>. Altogether, these data support a complex spatiotemporal regulation of TLR-mediated IFNα responses in pDCs, whose mechanisms are slowly starting to emerge. Further research is needed to improve our understanding and may help in the designed of better TLR7/9 adjuvants.

### **2.3. pDC-specific inhibitory receptors**

pDC express multiple inhibitory receptors that are crucial for the attenuation of TLR-induced production of IFNα and other cytokines, including BDCA2, FcεRIα and ILT7 which signal through a common pathway involving FcεRIγ or DNAX activation protein 12 (Dap12), spleen tyrosine kinase (Syk), and B cell linker (BLNK)<sup>17</sup>. Knockdown of FcεRIγ and Syk in the pDC-like cell line Gen2.2 enhances TLR9-mediated type I IFN production and reverses ITAM-triggered inhibition<sup>17</sup>. These inhibitory receptors provide a negative feedback to pDCs by confirming a successful IFN signal as exemplified by the receptor/ligand couple ILT7/BST-2 in which BST-2 is an IFN-inducible membrane protein that directly restricts viral replication. HIV-1 has been shown to inhibit pDC function by binding to BDCA2<sup>12</sup>. Engagement of FcεRIα by IgE leads to the inhibition of CpG DNA-induced type I IFN production by pDCs via FcεRIγ<sup>17</sup>. However, the Dap12 signaling pathway has also been involved in positive signaling for type I IFN production by pDCs in the context of PDC-triggering receptor expressed on myeloid cells (TREM)/PlexinA1/Dap12, which is activated preferentially on TLR-stimulated pDCs<sup>17</sup>, suggesting that these pathways are not universally suppressive for pDCs. In parallel, it was shown that low avidity ligation of the ITAM-associated FcαRI results in the translocation of FcαRI and SH2 domain containing tyrosine phosphatase 1 (SHP-1) to membrane lipid rafts and trafficking to an inhibitory intracellular compartment where signaling molecules are deactivated by SHP1<sup>17</sup>. In contrast, high-avidity interactions are generally thought to result in cell activation. Nevertheless, in pDCs, high-avidity interactions of ITAM-containing receptors lead to the attenuation of TLR-induced type I IFN production. Therefore, how ITAM signaling intersects and negatively regulates TLR7/9-mediated responses in pDCs remains elusive. As such, in addition to the negative feedback loop of type I IFN

mentioned, other mechanisms exist to also curtail pDC responsiveness.



**Figure 4: Regulation of TLR7/9 signaling in pDCs**

The pDC TLR7/9 pathway is tightly regulated. First, the processing of TLR7/9 involves multiple molecules. Ligand binding to TLR7/9 leads to the activation of the MyD88 complex. Downstream signaling depends on the localization of TLR7/9 at the time of stimulation. Activation in the early endosomes favors IFN $\alpha$  production while activation in the late endosome results in the transcription of proinflammatory cytokines such as TNF $\alpha$  and IL-6. IRF7 activation requires both its phosphorylation and K63-polyubiquitination. IRF7 can be regulated at multiple levels. Ligation of pDC specific surface receptors can either further enhance or repress IFN $\alpha$  production.

*Adapted from [17]*

### 3. The interferon regulatory factor 5

#### 3.1. Alternative promoter splicing of IRF5 Gene

*IRF5* gene is located on chromosome 7q32 and is composed of 8 coding exons and four non-coding first exons, which are alternatively used. Figure 5 illustrates *IRF5* gene structure. The start codon for IRF5 is in exon 2,

therefore the use of different first exons does not directly alter the protein sequence. Instead the four alternative promoters are four pathways to make the same protein. The first exons are 1A, 1B, 1C, and 1D. Exons 1A, 1C and 1D are activated upon TLR7 stimulation<sup>80</sup>. Exon 1A promoter contains an interferon regulatory factor binding element (IRFE)<sup>56</sup> while exon 1C promoter contains an ISRE site and STAT2 binding site<sup>56,80</sup>. Consequently, its expression can be upregulated by type I IFNs<sup>81</sup>. The promoter upstream of exon 1A has the highest constitutive activity and confers the highest translation efficiency<sup>80,82</sup>. It has been shown that the ability of a cell to use and splice the 1B exon is independent of its promoter usage, which may be repressed by p53<sup>80</sup>.

### 3.2. IRF5 isoforms and cell-type specific expression pattern

Several IRF5 isoforms, with distinct cell-type specific expression, cellular localization and activity, are generated by alternative splicing<sup>56</sup>. There are nine different IRF5 variants, with V7, V8, V9 detected only in human cancers. pDCs express high constitutive levels of IRF5 transcripts and proteins<sup>35,36,83</sup>. *IRF5* is transcribed into four distinct alternatively spliced isoforms (V1, V2, V3, and V4) in human pDCs with V3 and V4 being the most predominant transcripts expressed in unstimulated pDCs, in contrast with *IRF5* V5, V6, and V1 been the most abundant isoforms in numerous cell types<sup>56</sup>. The variants V1, V2 and V3 exhibit different noncoding first exons and distinct insertion/deletion patterns in exon 6. Alternative splicing of *IRF5* exon 6, partially encoding for IRF5 Proline (P), glutamic acid (E), serine (S), and threonine (T)-rich (PEST) domain, results in expression of IRF5 isoforms with increased stability and hence activity<sup>84</sup>. Transcripts associated with Ex1V2 were either very low (monocytes, NK, and B cells) or nearly undetectable (PBMCs, pDCs, and T cells). Ex1V1/Exon 1A transcripts were detected in all types of unstimulated cells (PBMCs, monocytes, NK, B cells and pDCs) except T cells. Ex1V3 transcripts were detected in unstimulated pDCs, monocytes, and B cells. Stimulation with IFN $\alpha$  significantly up-regulated the levels of Ex1V3/Exon 1c transcripts<sup>56</sup>. IRF5 V3 and V4 are the primary inducers of IFN $\alpha$  while V2

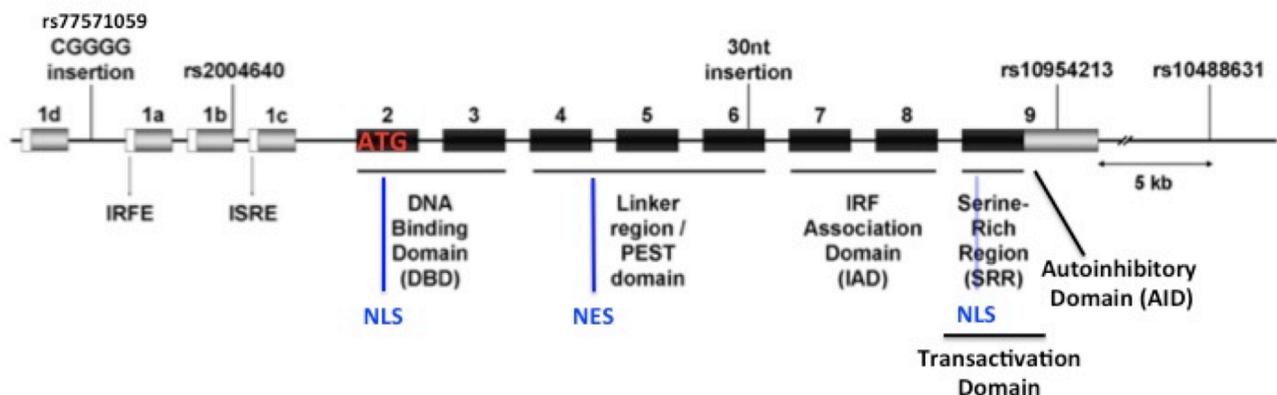


Figure 5: *IRF5* gene structure

Coding exons are represented as dark gray boxes and non-coding exons represented as light gray boxes. Promoter regions are represented as white boxes. IRF5 polymorphisms are indicated above the diagram, while protein domains are indicated below the diagram with corresponding coding exons.

Adapted from [93]

and V6 induce low levels of IFN $\beta$ . The majority of IRF5 isoforms does not differ in their DNA binding sites, but differ in the interaction domain. IRF5 isoforms have therefore different cell-type specific pattern and different IFN $\alpha$ -inducing capacities.

Interestingly, a large number of novel IRF5 isoforms originated by alternative splicing is exclusively expressed by SLE patients as compared to healthy donors<sup>85</sup>. For example, enhanced levels of IRF5 transcripts bearing exon 1D were observed in SLE patients as compared to healthy controls, suggesting that this exon may be specifically used in this disease context<sup>86</sup>. There is a CCCTC binding factor (CTCF) binding site upstream of exon 1D, which may contribute to silence the IRF5 gene by preventing its hypermethylation<sup>86</sup>. Importantly, there are important differences in splice patterns of murine versus human *IRF5*, which may bias the translation of results from one species to another<sup>56</sup>. Spliced variants of *IRF5* were identified only in human cells, while in inbred strains of mice, *IRF5* encodes for a dominant unspliced transcript.

### 3.3. IRF5 activation

IRF5 protein is a 60-63 kDa polypeptide with a N-terminal DNA binding domain (DBD), characterized by the presence of five tryptophan residues and folded in a winged Helix-Turn-Helix conformation, and a C-terminal IRF association domain (IAD) conserved in all members from IRF3 through IRF9 and important in mediating interaction with transcriptional co-activators<sup>87,88</sup>. The central region connecting the DBD and the IAD is a PEST domain, normally present in proteins characterized by a rapid turnover<sup>89,90</sup>. IRF5 is activated upon TLR7 stimulation<sup>83,91,92</sup> but not by TLR3 signaling pathway<sup>83</sup>. IRF5 is activated by NDV, vesicular stomatitis virus (VSV), and herpes simplex virus (HSV) type 1. In contrast, the Sendai virus and double-stranded RNA (dsRNA) polyIC activate IRF3 and IRF7 but not IRF5<sup>81</sup>. IRF5 has therefore a restricted activation pattern. It is activated in a virus-specific manner, and not by dsRNA<sup>81,91</sup>. IRF5 can also undergo nuclear translocation upon DNA damage<sup>93</sup>.

In contrast to IRF3 and IRF7, IRF5 can be detected in the nucleus of uninfected cells, which could result from the presence of two nuclear localization sequences (NLS), necessary elements for virus-induced nuclear translocation<sup>81,91</sup>. The 3' NLS is exposed and responsible for the transactivation activity of IRF5 in uninfected cells, whereas the 5' NLS is masked either by an intramolecular interaction or by association with another protein. Residues 150-160 of IRF5 function as an authentic nuclear export sequence (NES) that is responsible for the cytoplasmic localization of IRF5 in unstimulated cells<sup>84</sup>. Both NLSs and NES in IRF5 are constitutively active, but nuclear export is dominant in unstimulated cells. In addition, IRF5 transcriptional activity is blocked in unstimulated cells by constitutive photomorphogenesis 9 (COP9) signalosome (CSN), which further prevent IRF5 proteosomal degradation<sup>94</sup>. Similarly to other members of the IRFs, IRF5 requires post-translational modifications for its nuclear translocation and proper transcriptional activity. Similarly to IRF7, IRF5 activation requires MyD88, IRAK1 and TRAF6 in the TLR7 signaling pathway<sup>83,95</sup>. Phosphorylation of serine residues 471 and 486 in the C-terminal region of IRF5 results in the exposure of the 5' NLS, IRF5 nuclear accumulation<sup>87</sup> and

activation of *IFNA* promoters<sup>81,84</sup>. Phosphorylation of the Ser/Thr residues within NES motif may lead to IRF5 nuclear translocation<sup>84</sup>. IKK $\epsilon$ /TBK1 stimulates IRF5 dimerization but do not induce nuclear translocation of IRF5. IKK $\epsilon$ /TBK1 may be responsible for the phosphorylation of the C-terminal sites containing the IAD<sup>96</sup>. IKK $\beta$  was shown to phosphorylate IRF5 at Ser462 *in vitro*, inducing its dimerization and nuclear translocation<sup>97</sup>. In opposite to IRF7, IKK $\alpha$ -mediated phosphorylation of IRF5 negatively regulates its function<sup>98</sup>. Upon binding to IRF5, TRIM28 acts as a docking site for SET domain bifurcated 1 (SETDB1), therefore facilitating reversion to a closed chromatin state<sup>99</sup>. Such mechanism may represent a negative feedback loop to prevent excessive inflammatory response. IRF5 stability is negatively regulated by TRIM21-mediated K48- and K63-linked polyubiquitination upon TLR7 stimulation, leading to the inhibition of *IFNA* transcription<sup>100</sup>. Interestingly, IRF5 variants missing the first 48 nucleotides of the PEST domain-encoding region are resistant to TRIM21-mediated degradation<sup>100</sup>. The group of Betsy Barnes reported that IRF5 binds constitutively to the endogenous *IFNA* promoters in a cell type-dependent manner, which may be related to the levels of IRF5 and the isoform expressed<sup>88</sup>. In uninfected cells, the *IFNA* transcription is silenced by IRF5 interaction with the corepressors HDAC1, Sin3a, and SMRT<sup>88</sup>. Virus infection also triggers the export of SMRT out of the nucleus, leading to the association of IRF5 with coactivator proteins p300/CBP and acetylation of histones resulting in gene transcription<sup>88</sup>. Altogether, these data suggest that IRF5 is regulated both by mechanisms common to the regulation of IRF family members but also by distinct ones. It is noteworthy to point that the overwhelming majority of studies focusing on IRF5 regulation originated from one single group, the team from Betsy Barnes and Patricia Fitzgerald-Bocarsly.

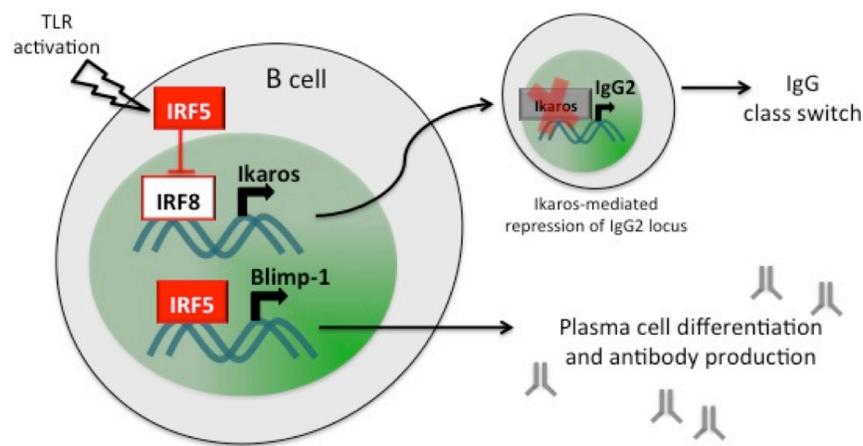
### 3.4. IRF5 Polymorphism

IRF5 polymorphisms have been associated with SLE in distinct populations with varying ethnic ancestries and through genome-wide association studies (GWAS)<sup>101,102</sup>. Four IRF5 polymorphisms have been identified as functional polymorphisms<sup>93</sup>. One polymorphism in the IRF5 coding region consisting in a 30 nucleotides insertion in the central region may lead to IRF5 isoforms with altered stability. Two polymorphisms in the promoter or untranslated regions (UTR) of IRF5 may have a direct effect on IRF5 expression: a single nucleotide polymorphism (SNP) near exon 1B called rs2004640 and a copy-number variant near exon 1A called rs77571059. The risk allele (T) of rs2004640 results in the creation of a splicing donor site allowing for correct splicing of exon 1B onto exon 2<sup>103</sup>. The rs77571059 polymorphism is an insertion/deletion (indel) of 5bp, and is commonly referred to as a CGGGG indel. The CGGGG indel (rs77571059) is found 64bp upstream of the transcription start site for exon 1A. Each allele has either three or four copies of the CGGGG repeat sequence. The 4-times copy-number variant allows binding of additional specificity protein 1 (SP1) transcription factor and results in increased IRF5 expression in PBMCs<sup>93</sup>. This 4-times variant is associated with SLE and increase in the transcription of the proinflammatory cytokines TNF $\alpha$ , IL12p40, IL-8, IL-1 $\beta$ , and IL-10. The CGGGG insertion is in

linkage disequilibrium with two rs2004640/rs10954213 SNPs<sup>103</sup>. The risk allele (A) of rs10954213 SNP situated in the 3' UTR lead to the expression of a shorter and more stable mRNA<sup>103</sup>. Unique IRF5 isoforms may arise from specific haplotype<sup>82</sup>. Importantly, the presence of the IRF5 risk haplotype correlates with enhanced IRF5 transcriptional activity, as demonstrated by the presence of increased levels of circulating IFN $\alpha$  in the serum of SLE patients carrying IRF5 risk haplotypes compared to patients carrying neutral or protective haplotypes<sup>104</sup>. In addition, the expression profile of genes known to be activated downstream of TLR, IFN receptor and B cell receptor (BCR) pathways are different in B cells from SLE patients carrying risk or protective IRF5 haplotypes<sup>93</sup>. IRF5 polymorphisms have been associated with a large number of autoimmune conditions such as primary Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease and Crohn's disease<sup>93</sup>, suggesting a more global pathogenic role of IRF5.

### 3.5. IRF5 functions

In addition to production of type I IFN, IRF5 is involved in several immune modulating functions including transcriptional regulation, antibody production and apoptosis. IRF5 is involved in the transcription of IL-12, IL-17, IL-23 and TNF $\alpha$  genes in humans<sup>93</sup>. Of note, those cytokines are also elevated in plasma from SLE patients as compared to healthy controls and correlate with disease activity<sup>93</sup>. It has also been demonstrated that IRF5 is key transcription factors involved in differentiation of macrophages towards an M1 phenotype<sup>93</sup>.



**Figure 6: Role of IRF5 in the modulation of B cell function**

IRF5 induces Blimp-1 transcription, therefore promoting B cell differentiation and plasma cell maturation by transcriptional control of the Blimp-1 gene. TLR-activated IRF5 inhibits IRF8-mediated Ikaros transcription. Ikaros represses the IgG2 locus. Its inhibition therefore favors IgG2 production and IgG class switching.

*Adapted from [93]*

IRF5 also controls B cell functions through two main mechanisms as shown in Figure 6. Importantly, studies in *IRF5*<sup>-/-</sup> mice and the effects attributed to *IRF5* deficiency may have been biased by the presence of a spontaneous mutation in a subpopulation of *IRF5*<sup>-/-</sup> mice of the dedicator of cytokinesis 2 (*Dock2*) gene, which is necessary for marginal zone B cell development and pDCs homing and function<sup>93</sup>. However, recent studies in *IRF5*<sup>-/-</sup> mice carrying a functional *Dock2* gene have confirmed that IRF5 deficiency greatly reduces the production of type I IFNs by pDCs<sup>105</sup> and that IRF5 is required for effective plasma cell generation but not for B cell development up until the splenic mature B cell stage<sup>93</sup>. IRF5 regulates transcription of the B lymphocyte-induced maturation protein (*Blimp-1*), which is critical for differentiation of B cells to plasma cells and IgG secretion<sup>93</sup>. IRF5 also indirectly controls pathogenic IgG class switching<sup>93</sup>. MyD88-activated IRF5 inhibits IRF8-mediated Ikaros expression, which during normal B cell maturation represses the IgG2a/c loci. The role of IRF5 on B cells also has clinical relevance in SLE pathogenesis<sup>93</sup>.

Furthermore, IRF5 is involved in multiple apoptotic pathways, including DNA damage-induced apoptosis and the DR/TRAIL-induced apoptotic pathway<sup>93</sup>. Apoptosis is essential for the development and maintenance of cellular homeostasis of the immune system, including selection of lymphocytes with appropriate T cell receptor (TCR) during development and decline of an immune response. Aberrations in genes encoding pro- or anti-apoptotic proteins have been implicated in the initiation of conditions such as immunodeficiency, autoimmunity and cancer. Importantly, IRF5 role in Fas-mediated apoptosis is cell-specific with a pro-apoptotic role in hepatocytes and DC but not in thymocytes and mouse embryonic fibroblasts (MEFs)<sup>93</sup>. Viral infection of cells overexpressing IRF5 results in the upregulation of a large number of genes involved in the apoptotic pathway, while virus-induced apoptosis is reduced in fibroblast from *IRF5*<sup>-/-</sup> mice<sup>93</sup>. IRF5 is pro-apoptotic in a p53-independent manner<sup>93</sup>. However, p53 has also been shown to activate IRF5 via a p53 enhancer site in exon2 of *IRF5*<sup>93</sup>. Overall, IRF5 governs various cellular functions.

#### **4. Central role of the interferon regulatory factors family in the regulation pDC TLR7/9 responses**

The IRFs family plays a crucial role in IFN production, with its members exhibiting various and even opposing functions<sup>106</sup>. As mentioned previously, IRF7 has a central role in the transcription of IFN $\alpha$  and as such is subject to multiple regulatory mechanisms notably via post-translation modifications. The translational repressors 4EBP-1 and 4EBP-2 repress the translation of IRF7 mRNA, while the mRNA-decapping protein 2 (*Dcp2*) promotes its degradation<sup>17</sup>. The tripartite motif-containing protein (*TRIM*) 28 and *PIAS1* can increase SUMOylation of IRF7, leading to the transcriptional repression of type I IFN genes<sup>17</sup>. Contrary to K63-linked polyubiquitination which is involved in IRF7 activation, K48-linked polyubiquitination leads to its proteasome-dependent degradation and can be mediated by the ubiquitin E3 ligase *RAUL* and *TRIM21* (also known as *Ro52*)<sup>17</sup>. However, their roles in

TLR7/9-induced responses in pDCs need further studies. IRF7 binds to its own promoter in a complex formed by FOXO3, nuclear corepressor 2 (NCOR2) and histone deacetylase (HDAC) 3 leading to IRF7 auto-regulation of its gene expression. Upon type I IFN stimulation, FOXO3 is degraded via the activation of the PI3K/Akt pathway in order to allow for sufficiently high level of IRF7 for the maximal antiviral response<sup>69</sup>. IRF7 is therefore under tight control.

IRF2 and IRF4 are two negative regulators of type I IFN signaling in pDCs. IRF2 antagonizes the type I IFN induced interferon-stimulated gene factor 3<sup>17</sup>. But IRF2 can also function as a transcriptional activator of certain genes through ISRE-like sequences<sup>17</sup>. It positively regulates RIG-I expression in B cells from HCV infected patients<sup>107</sup>. IRF4 selectively inhibits IRF5-dependent proinflammatory genes through competing with IRF5 for MyD88 interaction in a cell type specific manner<sup>17</sup>. IRF8 plays a significant role in magnifying the second phase of IFN transcription by prolonging binding of basic transcription machinery to the IFN promoters and also contributes to pDC development<sup>2,17</sup>.

Early works using different models showed contradictory results regarding the role of IRF5 in the transcription of type I IFN genes. IRF5 or IRF7 were shown to be sufficient for stimulation of *IFNA* gene expression in virus-infected human fibroblast cells<sup>108</sup>. In contrast, Takaoka and colleagues showed that the induction of IFN $\alpha$  by spleen-derived pDC upon CpG-A stimulation was normal or even enhanced in *IRF5*<sup>-/-</sup> pDCs, indicating that IRF5 does not participate in type I IFN induction upon TLR9 stimulation<sup>109</sup>. Instead, IRF5 was shown to be involved downstream of the TLR3/4/5/7-MyD88 signaling pathway for gene induction of proinflammatory cytokines, such as IL-6, IL-12 and TNF $\alpha$ <sup>109</sup>. IRF5 was shown to only weakly stimulate type I IFN promoter activities<sup>84</sup>. *IRF5*<sup>-/-</sup> mice appeared highly vulnerable to viral infections, accompanied by a decrease in type I IFN induction in the sera, suggesting that IRF5 is important for antiviral immunity<sup>110</sup>. Recently, IRF5 was shown to participate in IFN $\beta$  transcription upon TLR9 stimulation<sup>111</sup>. IRF5 recognizes the consensus sequence KAAAGGAAAGCnAAAAGTGA<sup>88</sup>. The group of Betsy Barnes contributed largely to emphasize the role of IRF5 in the transcription of type I IFN, notably in pDCs. IRF5 was shown to form homo- and heterodimers with different IRFs, resulting in differential effect on the assembly of the IFN $\alpha$  enhanceosome and subsequent differential regulation of transcriptional activity of *IFNA* genes<sup>91</sup>. Indeed, the presence of IRF5/IRF7 heterodimer in Newcastle disease virus (NDV)-infected cells inhibited the *IFNA1* promoter while IRF3/IRF5 heterodimer activates the *IFNA1* promoter<sup>81</sup>. IRF5 could therefore act as both an activator and a repressor of IFN gene expression depending on the IRF-interacting partner. They suggested that the roles of IRF5 and IRF7 are non-redundant and supported a model in which the relative levels of IRF5 and IRF7 expressed in infected cells, as well as their distinct virus-specific activation, can determine the distinct profile of IFN $\alpha$  subtypes induced<sup>95,112</sup>. IRF5 may also be involved in the repression of *IFNA* gene expression<sup>92</sup>. More recent work has demonstrated that IRF5 can constitutively binds to the endogenous *IFNA* promoters in a cell-type specificity manner and may dependent on the levels of IRF5 expressed and possibly the isoform expressed<sup>88</sup>. IRF5 interacts with both HATs and HDACs, along with other corepressor proteins. However, these interactions were, in part, dependent on

IRF5 post-translational modification in response to virus. Ectopic expression of IRF5 enabled type I IFN production in response to TLR7 signaling, whereas knockdown of IRF5 by small interfering RNA (siRNA) reduced type I IFN induction in response to the synthetic TLR7 ligand, R848<sup>83</sup>. These studies differed in the cell type and stimulants used, suggesting that the impact of IRF5 on type I IFNs transcription may be cell-type- and stimulus-specific.

Importantly, targeting of IRFs by viruses including HIV-1 represents a winning strategy to increase virus replication and spread and establishment of viral persistence and latency<sup>113</sup>. It was shown that HIV-1 exploits IRF7 in pDCs to stimulate a persistent IFN $\alpha$  production. Individuals with IRF7 polymorphisms have a lower production of pDC-derived IFN $\alpha$  in response to HIV-1 infection<sup>114</sup>. Recently, IRF5 was shown to be a target of the HIV-2 protein Vpx in cDCs leading to the inhibition of IRF5-mediated transactivation and TLR-dependent pro-inflammatory cytokines production in response to LPS<sup>115</sup>. Viral targeting of pathways upstream of type I IFN production are therefore important avenues to suppress antiviral immune responses.

Complex signaling pathways participate in the tight regulation of IFN $\alpha$  response by pDCs following detection of pathogens via TLR7/9. IRF5 is emerging as a critical regulator of type I IFNs responses in autoimmune diseases. Type I IFNs are crucial mediators of antiviral responses but also exhibit critical immunomodulatory functions. They have emerged as disease-promoting agents in multiple settings.

### III. Disease-promoting type I IFNs

#### 1. Protective role of type I IFNs

Early type I IFN response is critical to establish cell-intrinsic antimicrobial states in infected and neighboring cells, therefore limiting the spread of infectious agents such as viral pathogens <sup>42</sup>. Type I, II and III IFNs stimulate the transcription of ISGs. ISG-encoded proteins (ISP) restrict pathogen through multiple mechanisms such as inhibition of viral transcription, translation and replication, degradation of viral nucleic acids and alteration of cellular lipid metabolism <sup>42</sup>. Unique set of ISGs target distinct viruses. Of note, a subset of ISGs including myxovirus resistance gene (Mx) 1, protein kinase, IFN-inducible dsRNA dependent (PKR) (also known as eukaryotic translation initiation factor 2-alpha kinase 2; EIF2AK2), oligoadenylate synthetase (OAS) and ISG15 are so powerfully induced by type I IFNs that their expression (along with other type I IFN inducible genes) are often used as surrogate markers to gauge the activation of type I IFN signaling in multiple disease and infectious states and is cumulatively referred to as the 'interferon signature'. Mx1 is a potent cell-autonomous restriction factor with antiviral activity against a range of pathogenic viruses. Mx gene activation is fast and its protein product is detectable within 4 hours of activation. The virus inhibitory protein, viperin, is endoplasmic reticulum-associated, IFN-inducible and possesses antiviral activity against diverse families of DNA and RNA viruses, including HIV-1 and HCV <sup>113</sup>. The induction of viperin into HIV-1-infected cells disrupts lipid rafts, causing viperin redistribution to CD81 compartments, where HIV-1 buds in human macrophages <sup>116</sup>. . The ubiquitin-like modifiers ISG15 and the associated tightly regulated process known as 'ISGylation' are important regulators of host antiviral responses. ISG15 expression enhances IFN-mediated antiviral activity against many viruses, including HIV-1 <sup>117</sup>. The importance of this early IFN response to viral infection is highlighted by the fact that most viruses, including HCV and HIV-1 have evolved strategies to combat either IFN production or IFN action. Acute type I IFN signals also possess immune stimulatory potential required for clearance of infection including the promotion of NK cell-mediated lysis of infected cells, enhancement of T cell priming by DC, and maintenance of proliferation and activation of T cells <sup>42</sup>, as detailed previously in section I.2.3.

Despite its antiviral properties, increasing data are emerging regarding the detrimental role of type I IFNs. Bacterial infection subsequent to or together with viral infection has long been known to be a significant cause of mortality and morbidity in humans, particularly following influenza virus infection<sup>118</sup>. Recent works have highlighted the central role of type I IFNs in how viral infection sensitizes the host to bacterial infection<sup>118</sup>. Nevertheless, the response of IFNAR<sup>-/-</sup> mice to bacterial infections may vary depending on the species and route of infection <sup>119</sup> with IFNAR signaling protecting against extracellular bacteria such as *Streptococcus*, *Escherichia coli* and *Helicobacter* but being detrimental during intracellular bacterial infection such as during *Brucella*, *Salmonella*, *Chlamydia*, *Mycobacterium* infections <sup>119</sup>. More recently, the dogma of type I IFN-mediated protection during influenza virus infection was questioned by Davidson and colleagues who demonstrated that

type I IFNs contribute to morbidity and mortality through excessive innate immune responses leading to tissue damage<sup>120</sup>. The dichotomic role of type I IFNs is evident in chronic viral infections, such as HIV-1 and HCV, but has also been examined in other disease models such as lymphocytic choriomeningitis virus (LCMV) model and in SLE.

## 2. Dichotomic role of type I IFNs in viral infection: Insights from the LCMV model

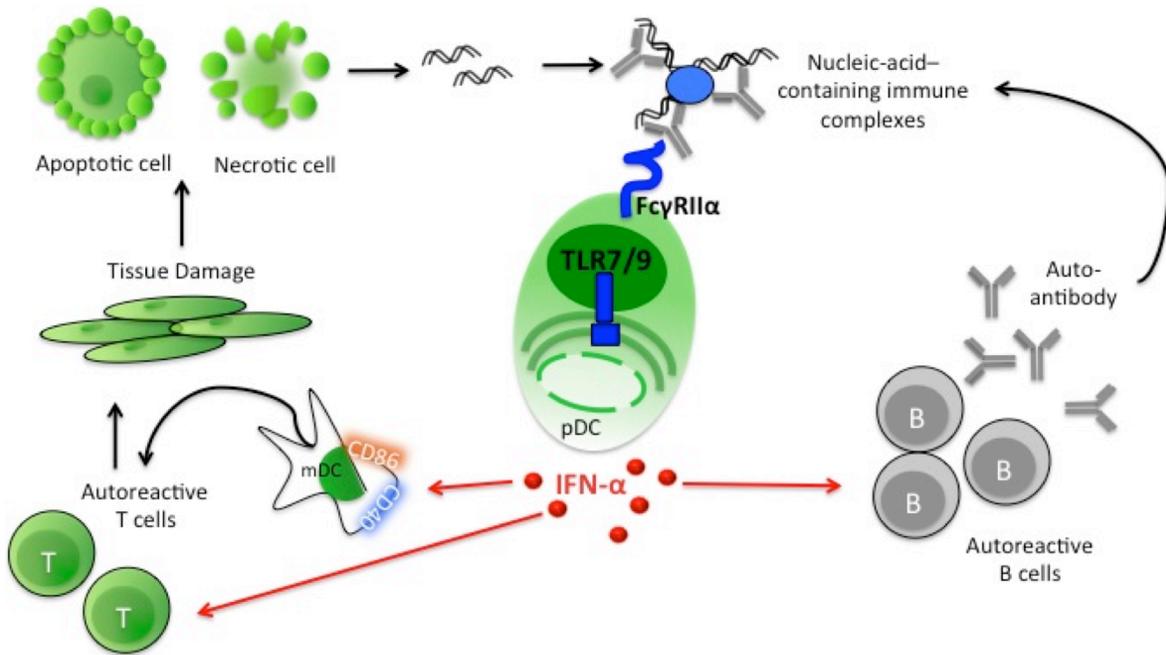
LCMV infection in mice is a very informative model to understand the dichotomic role of type I IFNs in viral infections. Indeed, two LCMV strains exist that initially trigger similarly robust type I IFN signaling<sup>121</sup>. As expected, removal of type I IFN signaling through genetic deletion of a subunit for the type 1 IFN receptor, or antibody-mediated blockade of the IFN receptor before infection increased viral replication and acute LCMV infection was no longer controlled<sup>121,122</sup>. The Armstrong strain (Arm) strain causes an acute infection that is cleared 8 days post-infection. Acute Arm infection resolution is accompanied by the rapid dissipation of ISGs expression. In opposite, the clone 13 (Cl13) strain induces persistent infection characterized by broad immune dysfunction<sup>123</sup>. pDCs are productively infected by Cl13 early in the infection<sup>122,123</sup>. ISGs expression and serum type I IFNs levels remained elevated in Cl13 infection<sup>121,122</sup>. The recent work from two independent laboratories have shed some light on why ongoing type I IFN signaling during chronic infection does not lead to viral control<sup>121,122</sup>.

Chronic type I IFN production can be rather deleterious to the immune system. Blockade of IFNAR1 highlighted several mechanisms by which type I IFN participate in Cl13 immunopathogenesis, including immune activation, sustained induction of immunosuppressive molecules IL-10 and PD-L1 that have been associated with viral persistence and T cell exhaustion in many chronic viral infections<sup>124</sup>, lymphoid tissue disorganization, and virus persistence<sup>121,122</sup>. Anti-IFNAR1 administration resulted leading to enhanced control of the persistent infection. Yet, how persistent LCMV infection is specifically controlled upon type I IFN blockade is not clear since enhancement of the two main effector mechanisms that directly eliminate virus infection (i.e., virus-specific CD8 T cells and antibody) were not observed. Instead, viral control seem to be influenced by type I IFN enhancement of the survival and/or maintenance of CD4+ T cells as well as increased IFN $\gamma$  expression and/or potency<sup>121</sup>. Inhibition of type I IFN signaling may have improved cellular interactions both spatially in the organized tissue and compositionally with the type of APCs present.

Altogether these data suggest that when virus cannot be controlled, acutely sustained type I IFN signaling induces immunosuppression that facilitates persistent virus infection<sup>121</sup>, a evolutionary mechanism that could aim to limiting immune-mediated pathology during infections where viral persistence is inevitable<sup>125</sup>. Indeed, “cytokine storms” (uncontrolled cytokine production and immune cell activation) or immune-mediated tissue destruction may turn out to be more damaging than beneficial in these settings. Interesting parallel can be drawn with the role of type I IFNs in the immunopathogenesis of chronic HIV-1 infection described in section

III.4.2. of this manuscript. It has been shown in other settings, that type I IFN immunosuppressive activity via IL-10 and/or PD-L1 induction can restrict immunopathology such as Flu infection <sup>42</sup> and by IFN $\beta$  treatment of patients with multiple sclerosis <sup>52</sup>. However, how the diverse downstream effects of type I IFN signaling are coordinated and which specific triggers are guiding distinct effects remains to be determined <sup>126</sup>.

### 3. Deleterious effect of type I IFNs in systemic lupus erythematosus



**Figure 7: Deleterious role of pDC-derived IFN $\alpha$  in SLE pathogenesis**

Nucleic acid-containing immune complexes are internalized by pDC in a Fc-receptor-dependent mechanism, leading to endosomal TLR activation and subsequent IFN $\alpha$  production. IFN $\alpha$ , together with other products of activated pDCs such as IL-6, promotes the differentiation of autoreactive B cells into plasma cells and the expansion autoreactive lymphocytes contributing to tissue damage. The newly secreted autoantibodies and nucleic acids released from necrotic cells provide new activating material for pDCs, further amplifying the pathogenic loop.

*Adapted from [127]*

Although IFN $\alpha$  production by pDCs has a protective role in some autoimmune diseases including inflammatory bowel disease and multiple sclerosis, its production is believed to contribute to the pathogenesis of other autoimmune diseases including SLE, Sjögren's syndrome, rheumatoid arthritis (RA) and psoriasis<sup>52,127</sup>. Figure 7 describes the central role of pDC-derived IFN $\alpha$  in SLE pathogenesis. Nucleic acids released from dying cells in the extracellular milieu are complexed with endogenous proteins such as LL37 and HMGB1, preventing them from being degraded by DNases and allowing their entry into endosomes. Such immune complexes are recognized by Fc $\gamma$ RII $\alpha$  on pDC and induce a strong IFN $\alpha$  response via TLR7/9 activation <sup>128</sup>. SLE patients exhibit high IFN $\alpha$  plasma levels and the activation of ISGs is correlated with disease activity and severity <sup>128</sup>. Over half of the genetic variants associated with lupus are linked to type I IFN production or signaling <sup>128</sup> and result in

enhanced type I IFN production or increased sensitivity to type I IFN<sup>128</sup>. SLE pathogenesis can also be linked to increased IL-6 production by activated pDCs. Together with IFN $\alpha$ , it promotes survival and differentiation of autoreactive B cells into autoantibody-secreting plasma cells. Taken together, these data show that excessive type I IFNs are involved in a pathogenic loop driving SLE disease.

## **IV. Detrimental role of IFN $\alpha$ in chronic HIV-1 infection**

HIV-1 infection is another prominent example of the detrimental role of IFN $\alpha$  in viral infections and the persistent challenges associated to therapeutic manipulation of IFN $\alpha$ .

### **1. Immune activation and inflammation as drivers of HIV-1 disease progression**

‘Immune activation’ describes cellular activation indicated by the expression of markers on immune cells including increased expression of HLADR, CD38, and Ki67 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as CD40 or CD80 on monocytes and DCs. Other markers of cellular activation exist including exhaustion markers that also regulate immune responses such as PD-1 or cytotoxic T lymphocyte-associated protein 4 (CTLA-4). Chronic immune activation is caused in part by antigen-specific T cell activation, but mostly by bystander activation resulting from the general activation of innate immune responses. ‘Inflammation’ refers to the production of cytokines and other mediators in response to a stimulus, resulting in redness, pain, heat, and swelling and subsequently immune cell recruitment and release of more inflammatory mediators. Immune activation and inflammation have been shown to be important predictors of HIV-1 disease progression<sup>129,130</sup> and are also predictive of higher set point plasma viral load<sup>131</sup> and even HIV-1 acquisition<sup>132</sup>.

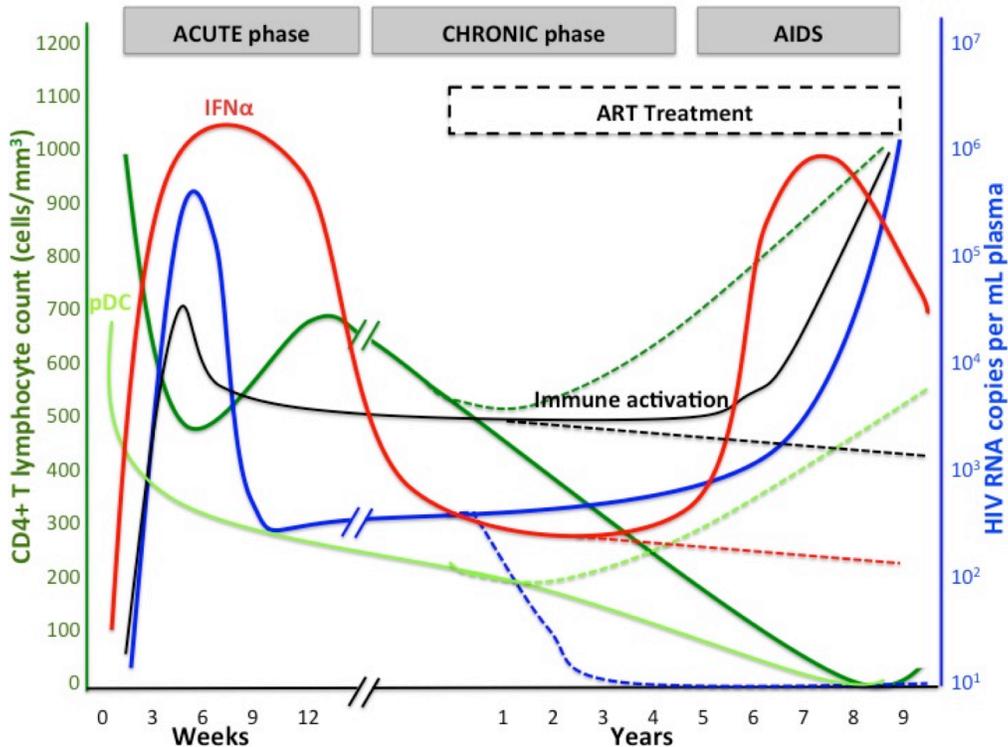
#### **1.1. Phases of HIV-1 infection**

According to the UNAIDS REPORT 2013, there were 35.2 million HIV-1-infected individuals worldwide in 2012. As a lentivirus, HIV-1 has ability to integrate into host’s genome, therefore causing persistent infection. In addition, HIV-1 main target cells are CD4<sup>+</sup> T cells, which have a vital role in the immune system. Thus, their destruction weakens the immune system’s ability to fight against infections and disease. The first phase of HIV-1 infection called ‘primary HIV-1 infection’ or ‘acute phase’ is characterized by a drop in CD4<sup>+</sup> T cell count, peripheral CD4<sup>+</sup> T cell numbers, and massive production and systemic spread of HIV-1, seeding a persisting viral reservoir in various organs, particularly the lymphoid organs such as the thymus, spleen, and lymph nodes. Acute HIV-1 infection induces a ‘cytokine storm’, striking cascade of systemic cytokine levels greater than seen in other acute viral infections and including IFN $\alpha$ , IP-10, TNF $\alpha$ , IL-10 and IFN $\gamma$ <sup>133</sup>. The peak in secretion of TNF $\alpha$  and IFN $\gamma$  occurs by seven to eight days post intravenous inoculation of simian immunodeficiency virus (SIV)<sup>134</sup>. For most cytokines, these changes are transient, lasting only weeks<sup>134</sup>. Extensive activation of both pro- and anti-inflammatory genes is also observed in LN tissues of subjects with acute HIV-1 infection, and this activation is more pronounced than at later stages of disease<sup>134</sup>. During this acute phase of infection, up to 70% of HIV-1-infected people suffer flu-like symptoms. Within 2-4 weeks after initial HIV-1 infection, adaptive immune responses consisting of CTL and antibodies help reduce the levels of viral replication to a so-called ‘viral setpoint’. The viral setpoint is highly predictive of the rate of HIV-1 disease progression, with individuals having

high viral setpoints being more likely to develop AIDS faster than those with low viral setpoints. Following the initial control of acute viremia, CD4<sup>+</sup> T cell counts begin to increase again, but, in most cases, do not return to pre-infection levels. The transition between the acute and chronic phases of the infection is accompanied by the partial shutdown of acute IFN $\alpha$  production, which may be related to activation-driven exhaustion<sup>135</sup> and increased renewal by BM-derived pDC precursors (non-functional Ki67 BM-derived pDCs) in the blood with no IFN $\alpha$ -producing capacity<sup>135</sup>. The subsequent 'asymptomatic' or 'chronic' phase of HIV-1 infection is characterized by lower levels of HIV-1 virus replication, persistent immune activation and cytokine production<sup>134</sup> and the absence of HIV-1-related symptoms. This phase can last for several years and even decades. Chronic HIV-1 infection is marked by a strong type I IFN signature<sup>12</sup>. While increased levels of IFN $\beta$  in HIV-1 infected individuals have been reported, the type I IFN signature in the peripheral blood of untreated, chronically HIV-1-infected individuals have been attributed to IFN $\alpha$  and no IFN $\beta$  has been found in LNs of HIV-1-infected patients (Hardy et al., 2013). Low levels of IFN $\alpha$  are produced during the chronic stage of pathogenic HIV-1/SIV infections until the late symptomatic stage. Certain IFN $\alpha$  subtypes, such as IFN $\alpha$ 2 and IFN $\alpha$ 6, may be specifically upregulated in both the acute and chronic phases of untreated disease<sup>12</sup>. Continuous CD4<sup>+</sup> T cell loss eventually leads to AIDS as the regenerative capacity of the immune system gradually decreases despite the excess of homeostatic cytokines. This last stage is characterized by a CD4<sup>+</sup> T cell count below 200 cells/mm<sup>3</sup> or by the development of one or more opportunistic infections. This last stage is also marked by high IFN $\alpha$  production. The kinetics of relevant clinical parameters during HIV-1 infection<sup>136</sup> are shown in Figure 8.

## **1.2. Clinical relevance of HIV-1-mediated immune activation and inflammation in the context of antiretroviral therapy**

The introduction of highly active antiretroviral therapy (HAART) in 1996 defined as the combination of three or more antiretroviral drugs marked a major breakthrough in the HIV-1 pandemic. It averted 6.6 million AIDS-related deaths worldwide from 1996 to 2012. It results in significantly higher CD4<sup>+</sup> T cell numbers, immune competence and reduces immune activation and occurrence of opportunistic infections, thus greatly improving life expectancy of treated patients<sup>137,138</sup>. Indeed, the median survival after diagnosis of AIDS was only about a year in the 1980s and early 1990s. Antiviral drugs are classified in five categories according to the where they target in HIV-1 life cycle: non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), entry or fusion inhibitors and integrase inhibitors. The most common HAART treatment consists of two NRTIs and one NNRTI or a protease inhibitor. We will simply refer to antiretroviral therapy (ART) in this manuscript when referring to combination ART.



**Figure 8: Evolution of clinical parameters during HIV-1 infection**

In acute infection, HIV-1 viral loads (blue line), IFN $\alpha$  plasma levels (red line) and T cell activation (black line) rapidly peak while CD4+ T cell count (dark green line) drops. In chronic infection, decrease in peripheral pDC count (light green line) parallels the decrease in CD4+ T cell count. Immune activation levels (black line) remain elevated. AIDS, characterized by CD4+ T cell count below 200 cells/mm<sup>3</sup>, is also associated with an increase in HIV-1 viral loads, IFN $\alpha$  plasma levels and immune activation levels. ART (dotted lines) allows to control HIV-1 viral load and to partially restore CD4+ T cell count. Abnormalities in IFN $\alpha$  plasma levels and immune activation levels may persist.

*Adapted from [136]*

Despite the success of antiretroviral therapy, elevated soluble biomarkers of inflammation and markers of T cell activation persist in treated HIV-1-infected individuals<sup>130</sup>. In particular, levels of sCD14 and sCD163, IL-6, hsCRP, D-dimer, IP-10 and IFN $\alpha$  remain elevated when compared with uninfected controls and even when HIV-1 viremia is fully suppressed<sup>130</sup>. The persistence of immune activation and inflammation under ART may increase non-AIDS-related morbidity and mortality, such as cardiovascular disease, kidney disease, liver disease, and non-AIDS-defining malignancies<sup>130</sup>. Monocytes activation in particular is associated with accelerated immune aging. It has been recently suggested that soluble markers of inflammation such as IL-6, soluble TNF receptors (sTNFR)-I/II or D-dimer but not T cell activation are associated with non-AIDS-defining events in HIV-1-infected persons with virological suppression<sup>139</sup>. Persistent immune activation and inflammation may be due to residual HIV-1 replication, loss of gut tissue integrity and microbial products translocation, persistent production of type I IFN co-infection with pathogens including cytomegalovirus (CMV), immune deregulation and lymphoid tissue fibrosis<sup>12,140</sup>. Although early ART initiation (i.e. while CD4<sup>+</sup> T cell counts are high) can result in reduced

inflammation and immune activation, antiretroviral drugs may also differentially affect inflammatory pathways<sup>141</sup>. As such, even in the setting of ART treatment and HIV-1 viral suppression, immune activation can still persist and contribute to disease progression. Activation of the innate immune response, directly or indirectly by HIV-1, contributes to this pro-inflammatory state.

## **2. Innate sensing of HIV-1 and IFN $\alpha$ response**

It has been shown that HIV-1 infection modulates the TLR responses by altering levels of TLR expression and by dysregulating responses of innate immune cells to TLR stimulation<sup>142</sup>.

### **2.1 TLR7-dependent recognition of HIV-1 by pDCs as a major driver of IFN $\alpha$ production in HIV-1 infection**

HIV-1 ssRNA activates the endosomal TLR7<sup>143,144</sup>. Indeed, HIV-1 encodes for multiple uridine-rich ligands for TLR7/8<sup>144</sup>. Endocytosis of HIV-1 virions by pDCs occurs via interaction of the envelope glycoprotein gp120 with CD4<sup>144-146</sup>. The degree of IFN $\alpha$  induction is correlated with the affinity of the virus to CD4<sup>12</sup>. However, co-receptor usage does not seem necessary for pDC activation by HIV-1<sup>144,147</sup>. Importantly, viral fusion and viral replication are not required for IFN $\alpha$  production<sup>12</sup>. pDCs produce high levels of IFN $\alpha$  upon stimulation by both live and inactivated, but fusion-competent, HIV-1 virions<sup>144</sup>. Retention of HIV-1 into early endosomes likely explains the high levels of IFN $\alpha$  secreted by pDCs in response to HIV-1<sup>12</sup>. Cell-associated virus also contributes to pDC activation, possibly through the uptake of HIV-1-containing apoptotic cell vesicles<sup>12</sup>, enhancing endosomal delivery of HIV-1 RNA to the TLR-containing endosomes. The mechanisms of HIV-1 trafficking in intracellular compartments, for free and cell-associated HIV-1 needs to be better characterized to understand how pDCs respond to HIV-1<sup>148</sup>

Cytosolic sensing of HIV-1 has not been shown to significantly contribute to IFN $\alpha$  secretion in pDCs<sup>12</sup>. The cytosolic exonuclease three prime repair exonuclease 1 (TREX1) has been shown to suppress cytosolic innate sensing in CD4<sup>+</sup> T cells and macrophages, and the HIV-1 Tat gene product can inhibit PKR<sup>12</sup>. These mechanisms may partly account for the predominance of TLR recognition of HIV-1 for IFN $\alpha$  secretion in pDCs<sup>12</sup>.

pDC are the major IFN $\alpha$  producers during HIV-1 infection, including in both lymphoid tissues and gut. Production of IFN $\alpha$  by other cell types such as splenic T and B cells<sup>149</sup> have been reported in chronic HIV-1 infection<sup>150</sup>. Importantly, this IFN $\alpha$  production may not depend on direct HIV-1 sensing, but rather on a bystander effect occurring in response to cytokines produced by HIV-1-activated pDCs<sup>12,148</sup>.

### **2.2. HIV-1 recognition by non-pDCs and contribution to IFN response and/or immune activation**

As mentioned above, HIV-1 encodes for TLR7/8 ligands. TLR8 is constitutively expressed by human cDCs and monocytes/macrophages. Recognition of HIV-1 genomic RNA by TLR8 induces NF- $\kappa$ B signaling. Recognition of HIV-1 RNA by TLR8 in monocytes leads to the formation of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome with activation of caspase 1 and IL-1 $\beta$  production<sup>151</sup>. The importance of TLR7 signaling in HIV-1 infection has been highlighted by genetic studies showing an association between genetic polymorphisms in TLR7 and differential disease outcome in HIV-1 infection<sup>152</sup>. In addition, Dominguez-Villar and colleagues have recently uncovered a surprising role for TLR7 in the elicitation of an anergic state in human CD4<sup>+</sup> T cells<sup>153</sup>. TLR7 engagement by synthetic ligands or HIV-1 leads to the induction of calcium flux and subsequent activation of nuclear factors of activated T cells, cytoplasmic, calcineurin-dependent 2 (NFATc2) resulting in the expression of anergy-related genes but also interfere with AP-1-dependent signals<sup>153</sup>. In addition, they showed that TLR7 engagement facilitated HIV-1 infection of CD4<sup>+</sup> T cells through the induction of an anergic state<sup>153</sup>. Further work is needed to appreciate the impact of TLR7 engagement in CD4<sup>+</sup> T cells *in vivo* in HIV-1 infection.

In the course of HIV-1 life cycle, several intermediates including intermediates resulting from abortive infection, are generated, such as double-stranded DNA (dsDNA). Those oligonucleotides can engage various sensors. SIV infection of monocyte-derived macrophages from macaque PBMCs demonstrate concomitant activation of an endosomal TLR pathway and the cytosolic RNA receptor melanoma differentiation associated gene 5 (Mda-5; also known as IFIH1) pathway<sup>154</sup>. It has recently been shown that the gamma-interferon-inducible protein 16 (IFI16) recognizes HIV-1 DNA intermediaries resulting from abortive infection of lymphoid quiescent CD4<sup>+</sup> T cells<sup>155</sup>, this activates caspase 1 signaling pathway leading to 'pyroptosis', an intensely inflammatory form of programmed cell death in which cytoplasmic contents and pro-inflammatory cytokines, are released and trigger the death of the vast majority of non-permissive, quiescent cells<sup>155,156</sup>. Levels of IFI16 in HIV-1-infected individuals were correlated to chronic immune activation<sup>157</sup>. In parallel, IFI16 activation triggers the activation of stimulator of interferon genes (STING)-dependent immune response via TBK1 and IRF3<sup>158</sup> and has been shown to stimulate the transcription of type I IFN and ISGs<sup>159</sup>. Sensing of HIV-1 intermediaries by IFI16 could therefore limit HIV-1 replication. To prevent IFI16 detection, HIV-1 exploits TREX1 in macrophages and lymphocytes, to maintain low levels of HIV-1 DNA during infection<sup>158</sup>. Similarly, HIV-1 has developed strategies to avoid retinoic acid inducible gene I (RIG-I; also known as DDX58) sensing upon productive infection<sup>158</sup>. RIG-I is targeted to lysosome to prevent immune responses against HIV-1.

Importantly, stimulation of innate immune cells is not limited to HIV-1-encoded ligands. HIV-1-mediated depletion of gut-associated CD4<sup>+</sup> T cells in early in HIV-1-infection contributes to an increase in microbial translocation (MT) through a compromised mucosal barrier. The resulting elevated systemic levels of microbial products such as LPS can stimulate a cascade of cytokine production via TLR4 stimulation<sup>160,161</sup> and are associated with a general increase in immune activation<sup>160</sup>. HIV-1 therefore directly or indirectly contributes to immune activation via the innate immune pathways including pDC production of type I IFN.

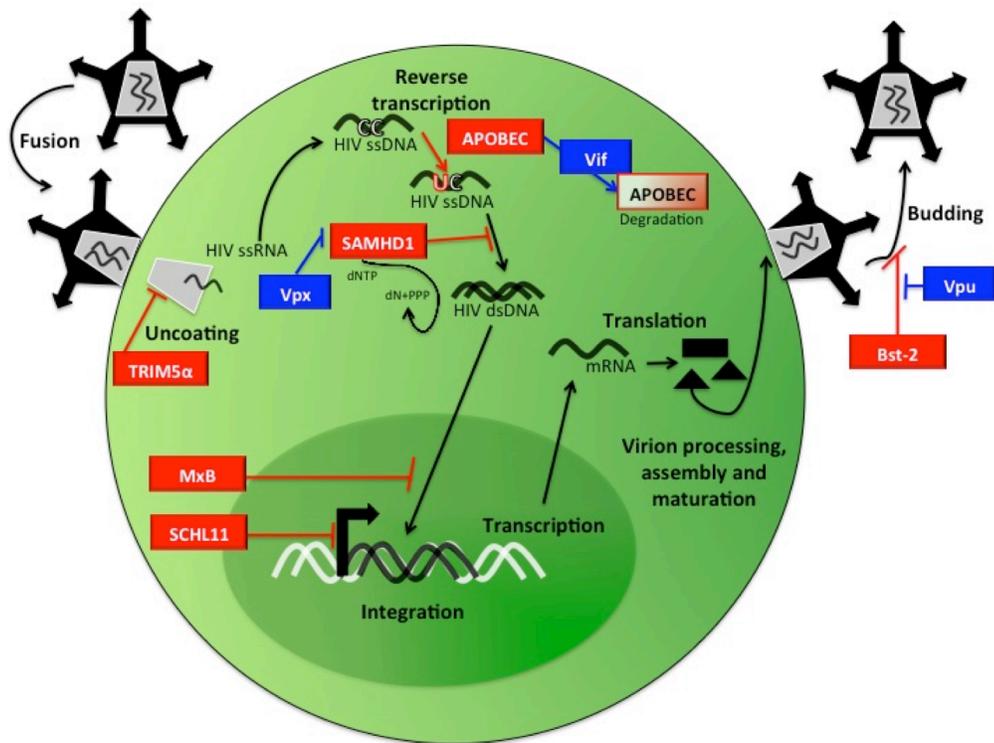
## 2.3. ISGs induction

ISGs are upregulated in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subpopulations<sup>162</sup>, monocytes and DCs<sup>162</sup> in untreated HIV-1 infection. In addition, ISGs levels positively correlate with HIV-1 viral load and inversely correlate with CD4<sup>+</sup> T cell counts<sup>162</sup>. ISG expression does not normalize with ART and elevated levels of ISGs (ISG56, IFI16 and IFI27) continue to be observed in CD4<sup>+</sup> and CD8<sup>+</sup> T cells isolated from HIV-1 patients on long-term (434 months) ART when compared with age-matched healthy donors<sup>163</sup>. As such although HIV-1 stimulated ISG production, after chronic HIV-1 infection is established, detectable viremia is not required for continued ISG production.

Host restriction factors can interfere with the production of new viruses by acting on different stages of the viral life cycle<sup>158</sup>. Many of them are induced by IFN<sup>158</sup>, but HIV-1 has developed strategies to counteract the actions of several ISG products as shown in Figure 9<sup>158</sup>. The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-3G and to a lesser extent APOBEC-3F and one allele form of APOBEC-3H can restrict HIV-1 post-entry blocking by triggering massive deamination of deoxycytidine to deoxyuridine within the retroviral minus (first)- strand cDNA, which impairs initiation and elongation rendering the virus nonviable within a single replicative cycle. The APOBEC3 family exhibit a strong inhibitory activity against HIV-1 *in vivo* but HIV-1 Vif has been shown to counteract their action in primary T cells by inducing its ubiquitin-mediated proteasomal degradation<sup>158</sup>. MXB (also referred as MX2), an interferon-induced protein, has recently been shown to reduce of nuclear accumulation and chromosomal integration of nascent viral complementary DNA<sup>158</sup>. Schlafen 11 (SCHL11) selectively inhibits the expression of viral proteins in a codon-usage-dependent manner<sup>164</sup> based on the bias toward an adenosine in the third position of the codon for many retroviruses. BST-2 blocks the release of diverse mammalian enveloped viruses and a wide array of retroviruses from several mammal host species, by directly tethering viral particles to the membranes of infected cells leading to virions internalization for degradation in lysosomes<sup>158</sup>. However, it may have a role in the promotion cell-to-cell transfer through the concentration of virions at the cell surface, therefore enhancing the formation of virological synapses<sup>158</sup>. BST-2 is antagonized by the HIV-1 accessory Vpu<sup>165,166</sup>. Besides, BST-2 is also a ligand for the receptor ILT7 found on human pDCs, acting as negative regulator of the production of IFN by pDCs in response to signaling downstream TLR7 and TLR9<sup>17</sup>. These are just some of the ISG products that are exploited by HIV-1 to evade IFN driven antiviral activities.

## 3. Dynamics of pDC in HIV-1 infection

### 3.1. Susceptibility to HIV-1 infection and depletion of pDCs in the peripheral blood in HIV-1 infection



**Figure 9: HIV-1 restriction factors**

Restriction factors are targeting different steps of HIV-1 life cycle, from uncoating to virions budding. HIV-1 proteins are hijacking their actions. Vif is mediating APOBEC proteasomal degradation. Vpu antagonizes Bst-2 effect on virions budding and Vpx prevents SMAHD1-mediated inhibition of HIV-1 reverse transcription. Adapted from [8]

The numbers of circulating pDCs and mDCs are reduced in untreated HIV-1 infection <sup>12</sup>. In fact, pDCs numbers correlate with CD4<sup>+</sup> T cell counts in both adults and children and their depletion is associated with high viral load (VL) <sup>12</sup>. The longitudinal kinetics of pDC levels have been rigorously defined in SIV-infected macaques. pDCs increase rapidly in blood during the first few days of infection, then rapidly decline to about half their baseline levels by the time of peak viremia (days 10–14 post infection). Nadir is attained 2 weeks after infection <sup>167</sup>. While pDCs numbers are partially restored with ART, persistent abnormalities persist despite treatment <sup>168-170</sup>. pDCs have been associated to HIV-1 disease progression and development of opportunistic infections <sup>171</sup>. Different mechanisms have been proposed to explain the reduced frequency of pDCs observed in the peripheral blood of HIV-1 infected patients, including cell death resulting from direct infection <sup>171</sup>. pDCs express HIV-1 co-receptors CD4, CXCR4, and CCR5 but not dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN). They can be infected at low level *in vivo* <sup>12</sup>. Recent work from the group of Nina Bhardwaj has highlighted the role of high levels of SAM domain and HD domain 1 (SAMHD1) in the blocking of productive infection in pDCs, in a similar way as cDCs <sup>172</sup>. A second virus, HIV-2, although less pathogenic, can also cause AIDS, encodes for the accessory protein, Vpx. Vpx induces the proteasomal degradation of SAMHD1, therefore allowing for DC infection by HIV-2. Blockade of type I IFN signaling in pDCs partially restored the ability of Vpx to induce

SAMHD1 degradation and caused the cells to become partially permissive to infection<sup>172</sup>. Of note, monocyte-derived DCs may not allow for proper HIV-2 entry and therefore cannot be efficiently infected by HIV-2 despite Vpx-mediated degradation of SAMHD1<sup>173</sup>. Neutralizing IFN modestly rescues HIV-1 infection of pDCs *in vitro*<sup>174</sup>, suggesting the existence of an IFN-independent mechanism to block HIV-1 replication in these cells. Due to the poor DC infectivity, it is unlikely that infection alone explains the reduced pDC frequencies observed. Evidence from non-human primates (NHP) model suggest that pDCs accumulate within lymphoid organs during early disease<sup>167</sup> and that this sequestering of pDCs accounts for the reduced levels detected in the periphery.

### **3.2. Rapid and significant recruitment of pDCs to lymph nodes**

HIV-1 sensing by pDCs induce the expression of LN homing markers CCR7, CD62L and CXCR3 leading to their trafficking to the LNs<sup>12</sup>. pDCs rapidly and massively migrate to LNs in acute SIV infection in both pathogenic and non-pathogenic hosts<sup>12</sup>. The expression of CXCL9 and CXCL10 is markedly increased in SIV infection of macaques. LN-homed pDCs are found in interfollicular areas in clusters. LNs are a major site of DC/T cell interactions as well as a major site of HIV-1 replication. Migration of pDCs to LNs during acute infection brings them into an environment rich with virus and virus-infected cells, facilitating cell-to-cell fusion and death by apoptosis and necrosis. Accumulation of activated pDCs in the lymphoid compartment could contribute significantly to generalized T cell activation. It was reported that IFN $\alpha$  produced by pDCs in LNs mediates LN-homed CD4<sup>+</sup> T cells hyperactivation and upregulation of ISGs<sup>146</sup>. pDCs appeared to be subsequently dying in the lymph nodes. It has been reported that about 4% of the LN-homed pDCs are infected in acute pathogenic SIV infection<sup>175</sup> and that pDCs from acutely SIV-infected LNs uniformly expressed the apoptotic marker CD95<sup>175</sup>, suggesting that activation-induced apoptosis and to a lesser extent direct virus infection accounts for pDCs death in LNs. pDCs also appeared to be depleted from LNs in chronically HIV-1-infected individuals and in advanced pathogenic SIV infection. The destruction of LN architecture may prevent the recruitment or retention of pDCs and therefore also contribute to pDCs depletion in later phases of HIV-1 infection. pDCs derived from non-pathogenic hosts may return to the circulation following the onset of the chronic phase<sup>176</sup>. pDCs produce high levels of IFN $\alpha$  in the LNs during both acute and chronic infections<sup>12,177</sup>, which may not be driven by active virus replication but rather by HIV-1 proteins trapped in the intercellular space of LNs. LN-homing pDCs express low levels of BDCA2, which may favor high IFN $\alpha$  production. The significant pDCs relocation to LN may be rescued with ART as shown in SIV model<sup>167</sup> but does not necessarily mean pDCs in the periphery return to pre-infection levels.

### **3.3. Rapid and long-lasting accumulation of pDC in the gut**

The gastrointestinal (GI) tract is a major site of HIV-1 and SIV replication and CD4<sup>+</sup> T cell depletion, particularly during the acute phase of disease. Recent works in NHP have shown that pDCs also traffic to the gut

mucosa in acute SIV infection and that pDCs relocation to the gut persists throughout the chronic phase of infection<sup>12</sup>. It has been suggested that pDCs may gain the ability to home to the gut even before they have emigrated from the BM<sup>178</sup>. Similarly, depletion of circulating pDCs observed in chronically untreated HIV-1 infected individuals was paralleled by their accumulation in the terminal ileum<sup>179</sup>. pDCs in gut-associated lymphoid tissue (GALT) can produce high levels of IFN $\alpha$  and TNF $\alpha$ <sup>178,179</sup>. Brenchley et al. showed a positive correlation of IFN $\alpha$  levels, LPS plasma levels, and the presence of activated CD8<sup>+</sup> T cells (defined as CD38<sup>+</sup> HLA-DR<sup>+</sup>) in chronically HIV-1-infected patients<sup>160</sup>. Such local inflammation of the gut mucosa, leading to a loss of mucosal integrity and subsequent bacterial translocation may drive systemic immune activation through both LPS and IFN $\alpha$  with excessive local release of IFN $\alpha$  in the GALT refueling serum levels. pDC numbers in the gut remain elevated despite ART treatment<sup>179</sup>. CD8<sup>+</sup> T cells also remain elevated in HIV-1-infected individuals during ART while CD4<sup>+</sup> T cell recovery appears slower in the gut than in the blood<sup>179</sup>. Possible reasons for the partial immune recovery in the GALT of HIV-1-infected subjects on ART include damaged tissue architecture and collagen deposition as well as continued low level HIV-1 replication in the GALT. As such, sequestering of pDCs in the gut and not the LN may explain the reduced peripheral pDC population observed.

### **3.4. Skewed maturation and persistently IFN $\alpha$ -secreting phenotype of pDCs in HIV-1 infection**

*In vitro* studies have shown that pDCs can be activated by HIV-1/SIV particles via TLR7, resulting in subsequent IFN $\alpha$  production<sup>144,145</sup><sup>12</sup>. pDCs are classically described as being refractory to IFN $\alpha$  production upon repeated stimulation with synthetic TLR7 or TLR9 agonists, which is thought to be a protective mechanism against excessive immune activation<sup>12</sup>. However, the group of Nina Bhardwaj showed that HIV-1 uniquely allows for persistent stimulation of pDCs, skewing them toward a partially matured and persistently IFN $\alpha$ -secreting phenotype<sup>10</sup>. Some studies have shown that pDCs from HIV-1 infected individuals may be less responsive to *in vitro* TLR7 and TLR9 stimulation<sup>148</sup>. This compromised functionality is thought to occur because of *in vivo* activation of pDCs. In the setting of HIV-1 replication, pDCs may be activated through different mechanisms: directly or indirectly by HIV-1, by microbial products such as LPS that may be released into systemic circulation through a breakdown of the gut mucosal barrier and through interactions with activated T cells by inflammatory cytokines<sup>148</sup>. Besides, pDCs from viremic HIV-1-infected patients express lower levels of CD4 on their surface than pDCs from uninfected controls. Decreased surface CD4 expression correlated with decreased IFN production in response to stimulation of pDCs with HIV-1<sup>180</sup>. Desensitization to type I IFN in HIV-1 infection is associated with reduced IFNAR expression<sup>181</sup>. Furthermore, defects in viral-induced IFN- $\alpha$  production by pDCs persist despite ART<sup>168-170</sup>.

pDC differentiation into functional APCs requires specific signals that are distinct from the signals that induce large amounts of type I IFN<sup>14</sup>. pDCs from viremic HIV-1 infected individuals have a partially activated phenotype. HIV-1-stimulated pDCs express low levels of co-stimulatory CD86 molecule and express IDO. The increased expression of CD86 and CD40 on blood DCs in viremic patients compared to healthy donors is directly

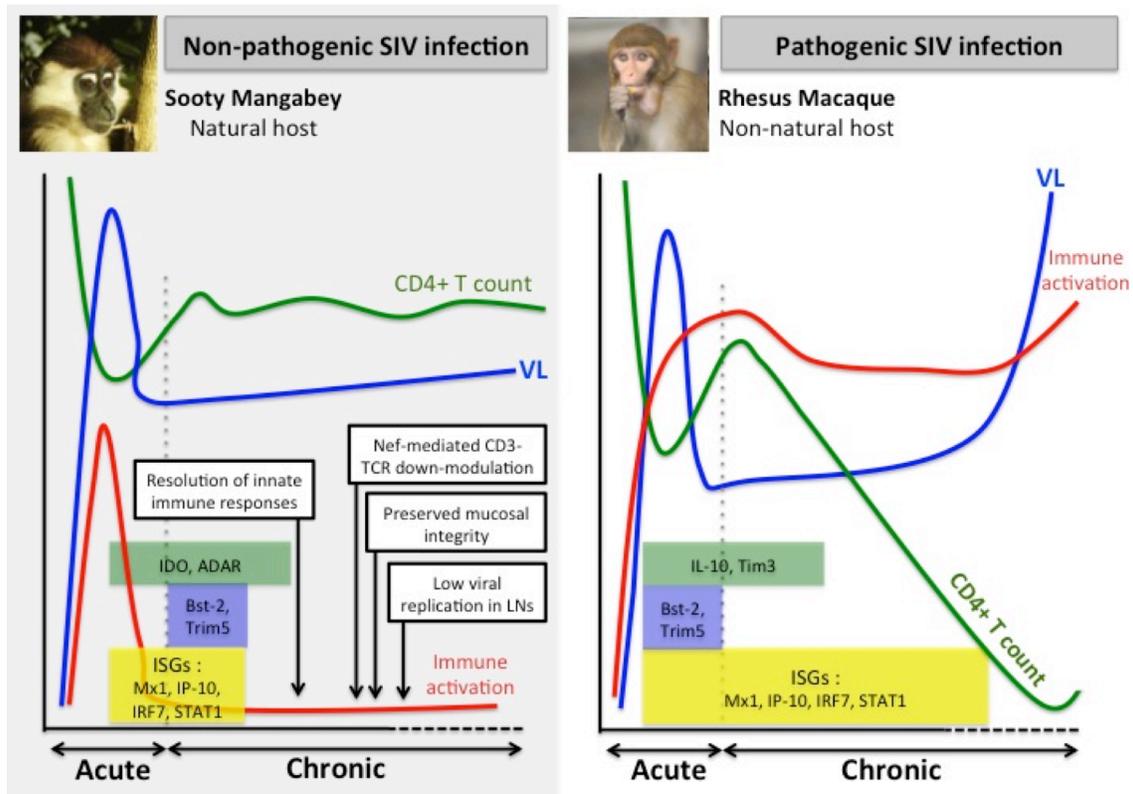
correlated with VL<sup>182</sup>. The maturation marker CD40 ligand has been shown to be necessary for virus replication, which could alter pDC viability. Incomplete maturation of DCs *in vivo* during HIV-1 infection may be due to weak stimulatory signal or a combination of signals given by different HIV-1 antigens, with some only partially activating DC and others inhibiting DC maturation. Besides, stimulation with HIV-1-infected activated T cells has been shown to induce a more complete activation phenotype than stimulation with HIV-1 particles *in vitro*<sup>183</sup>. In the absence of substantial viral replication, such as during ART, similar CD40 levels on pDCs and cDCs between treated subjects and healthy donors have been reported<sup>184</sup>. Partial maturation of pDCs and DCs in general may have significant consequences. It may lead to failure to generate new antigen-specific T cell responses while maintaining the ability to non-specific T cell activation. Co-culture experiments with T cells show that HIV-1-exposed pDCs are impaired in their ability to stimulate allogeneic T cell proliferation and instead it may induce apoptosis, possibly via production of inflammatory cytokines<sup>148</sup>. Nevertheless, pDCs differentiated into APC with the synthetic TLR7 agonist R837 are able to cross-present antigens from HIV-1-infected apoptotic cells to HIV-1-specific CD8<sup>+</sup> T lymphocytes<sup>12</sup>. This suggests that pDCs do not have an intrinsic defect in presentation and may be capable present viral antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HIV-1 infection once they are properly matured. Importantly, the partial activation state of circulating pDCs in viremic patients may not reflect the activation state of pDCs in other compartments. It is possible that pDC having reached full maturation are preferentially migrating to the lymphoid organs and therefore pDCs remaining in the blood could be those that have failed to achieve a sufficient maturation state.

Effective ART can correct some aspects of the chronic inflammatory response in HIV-1 infection, including partial restoration of plasma IFN $\alpha$  levels to normal<sup>185</sup>. Early ART may prevent chronic stimulation of the pDCs and allows the preservation of their functions. It was shown that prolonged ART initiated at the time of HIV-1 seroconversion is associated with a polyfunctional immunological T cell status that is similar to that of long-term nonprogressors<sup>186</sup>. However, conflicting data regarding ART treatment on both pDC function and type I IFN levels exist and needs to be examined further with greater scrutiny on type and timing of treatment.

#### **4. Role of type I IFN in HIV-1-mediated chronic immune activation**

Since 1991, high levels of plasma IFN $\alpha$  have been associated to disease progression during late-stage of HIV-1 infection. This has been followed by observations that elevated ISGs levels and immune activation are also associated to AIDS progression<sup>129</sup>. Our group showed that upon HIV-1-encoded TLR7/8 ligand stimulation higher IFN $\alpha$  production is observed in women than in men was correlated with an associated higher activation of CD8<sup>+</sup> T cells<sup>187</sup> and increased ISG levels<sup>162</sup>. While an important body of correlative data in humans have suggested a pathogenic role of IFN $\alpha$  in HIV-1-induced immune activation, studies in animal model allowing for more controlled settings have provided critical information this past decade.

#### 4.1. Persistent type I IFN signaling as a characteristics distinguishing pathogenic versus non-pathogenic SIV infection



**Figure 10 : IFN response and immune activation in pathogenic versus non-pathogenic SIV infection**

Despite high levels of viremia, levels of immune activation remain low in natural SIV hosts, preventing the occurrence of AIDS. Several mechanisms may be involved, including Nef-mediated CD3-TCR down-modulation, preserved mucosal integrity, low levels of virus replication in LNs and the resolution of innate immune responses. ISGs are rapidly downregulated in natural hosts while they persist throughout chronic infection in non-natural hosts. The pattern of expression of restriction factors such as Bst-2 and Trim5, and of immunomodulatory proteins such as IDO, ADAR or IL-10 differ between the two hosts.

*Adapted from [190]*

*Pictures from <http://www.quantum-conservation.org/ESB/sooty-mangabey.gif>;*

*[http://www.newswise.com/images/uploads/2006/02/10/fullsize/Rhesus-1\\_4.jpg](http://www.newswise.com/images/uploads/2006/02/10/fullsize/Rhesus-1_4.jpg)*

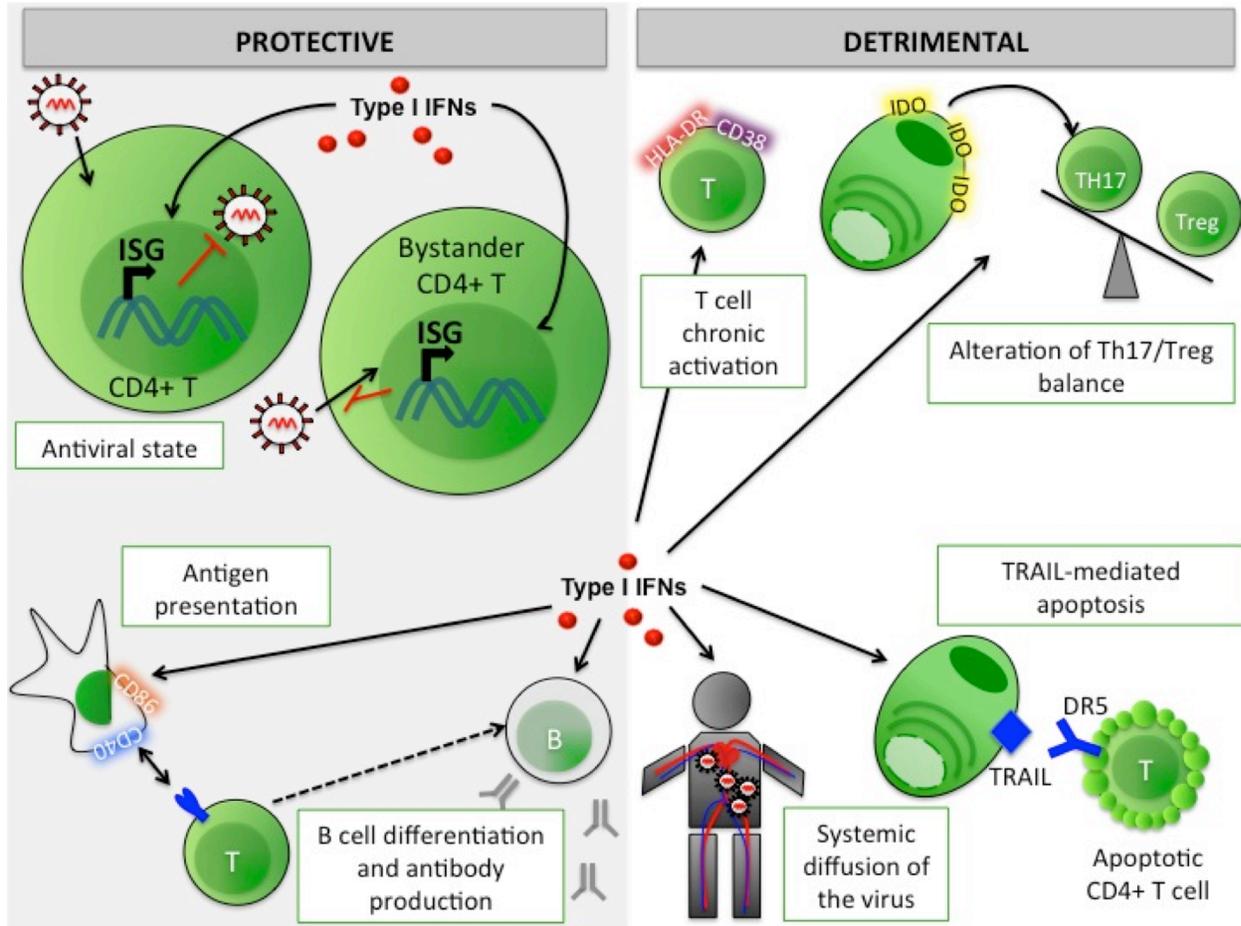
Fundamental clues regarding the role of IFN $\alpha$  as a driver of immune activation comes from NHP models of SIV infection. SIV infection in natural hosts such as sooty mangabeys (SM) and african green monkeys (AGM) or non-natural hosts such as rhesus macaques (RM) induce high viremia and significant loss of mucosal CD4<sup>+</sup> T cells during acute infection <sup>188</sup>. Pathogenic SIV infection observed in non-natural hosts is characterized by sustained depletion of peripheral and mucosal CD4<sup>+</sup> T cells, loss of gut mucosa and persistently high levels of proinflammatory cytokines and lymphocyte activation that culminate in disease progression and AIDS <sup>188</sup>. The rapid resolution of acute immune activation in the natural hosts is crucial for limiting long-term pathogenesis

<sup>189</sup>. Several mechanisms have been proposed including: (1) acquired resistance to activation of infected CD4<sup>+</sup> T cells after down-modulation of the cell surface CD3-TCR complex by the Nef protein of SIV<sub>simm</sub> and SIV<sub>agm</sub>; (2) preservation of mucosal immunity; (3) low levels of virus replication in LNs and other organized lymphoid tissues in chronic SIV infection of AGMs and SMs; and (4) differences in innate immune responses <sup>189,190</sup>. Despite robust virus replication, the key markers of innate immune activation, such as production of type I IFNs by pDCs, up-regulation of ISGs, and activation of macrophages and natural killer cells, are not elevated during chronic SIV infection of natural hosts <sup>189</sup>. It was first hypothesized that the pDC response to TLR7/9 signaling was defective in SIV natural hosts <sup>191</sup>. However, pDCs from acutely infected AGMs were capable to produce high levels of IFN $\alpha$  including after repeated stimulation, resulting in strong induction of ISGs <sup>192</sup>. Cells derived from natural hosts were neither refractory nor resistant to stimulation or re-stimulation, demonstrating a fully functional type I IFN pathway in natural hosts <sup>192</sup>. In addition, they display intact IRF7 function <sup>193</sup>. The recent study from Jacquelin et al. supports the hypothesis that the reduced levels of IFN $\alpha$  produced during acute infection in non-natural hosts is not responsible for the lower levels of immune activation observed in natural hosts. Indeed, artificial increase of IFN $\alpha$  levels through exogenous injection of type I IFN at the peak of type I IFN production in AGM did not alter their ISG profile nor promote immune activation <sup>194</sup>. Compelling transcriptomic evidences have shown that a key distinction between the non-pathogenic and pathogenic models comes from the extent and duration of the type I IFN response. Figure 10 illustrates the kinetics of IFN response and immune activation in pathogenic versus non-pathogenic SIV infection. While ISG expression persists during the chronic phase of SIV infection in non-natural hosts <sup>192</sup>, type I IFN production and ISGs expression are spontaneously downregulated in natural hosts 4–8 weeks post infection in both the peripheral blood and in LNs. This is evident in Harris et al. study, which demonstrates that AGM and SM begin to downregulate MxA responses by 28 days post-infection whereas RM maintained high levels of MxA in LNs <sup>195</sup>. The SIV model therefore highlights the higher type I IFN and subsequent ISG levels as a possible mechanism for the differences in immune activation between natural and non-natural hosts of SIV.

The regulatory complexity of the type I IFN pathway and its overall effects on target cells make pinpointing a resolution mechanism challenging. A role for immunomodulatory proteins such as IDO and adenosine deaminase acting on RNA (ADAR) has been suggested <sup>196</sup>. The rapid down-modulation of the innate immune activation in acute SIV infection has been shown to coincide with a rapid upregulation of PD-1 expression in LNs <sup>197</sup>. It has been speculated by Jacquelin et al. that the mechanisms controlling inflammation in natural hosts are in place before the transition of the acute to the chronic phase <sup>194</sup>. These mechanisms need to be more closely examined to understand how immune activation can be controlled.

## **4.2. Mechanisms underlying deleterious effect of IFN $\alpha$**

Chronic activation of type I IFN signaling is a pathological trait in SIV infection. Several mechanisms underlying the deleterious effect of persistent IFN $\alpha$  production in chronic HIV-1 infection have been uncovered. Figure 11 illustrates the dichotomous role of IFN $\alpha$  in HIV-1 infection.



**Figure 11 : Mechanisms underlying dichotomous role of IFN $\alpha$  in HIV-1 infection**

IFN $\alpha$  mediates protective effect that may be particularly important for the control of HIV-1 infection in acute phase (*left panel*). IFN $\alpha$  is responsible for the induction of antiviral responses in both infected CD4+ T cell and bystander cells. Type I IFNs also promote antigen presentation as well as B cell differentiation and antibody production. Type I IFNs participate however to the systemic diffusion of the virus by attracting activated cells at the mucosal site. In chronic infection, IFN $\alpha$  drives immunopathology (*right panel*), by upregulating T cell activation marker, altering the Th17/Treg balance through the induction of IDO and promoting TRAIL-mediated apoptosis of bystander CD4+ T cells.

#### 4.2.1 Increased dissemination of the virus by pDCs

In HIV-1 infection, pDCs are rapidly recruited at the mucosal site of infection (as early as 1 day post-infection), presumably through CCL20/MIP3 $\alpha$  secretion by endocervical epithelial cells <sup>198</sup>. SIV exploits pDCs to overcome the limited availability of susceptible target cells, resulting in the expansion of the initially infected founder cell populations and subsequent establishment of a self-propagating infection in secondary lymphoid

organs. Indeed, exposure of endocervical epithelium to the viral inoculum increases expression of MIP3 $\alpha$  to recruit pDCs, which, in turn produce MIP-1 $\alpha$  and MIP-1 $\beta$  and IFN $\alpha$  to recruit CCR5<sup>+</sup> targets<sup>199</sup>. Antiviral IFN $\alpha$  responses induced by intravaginal application of TLR7 or 9 agonists are overcome by the virus and inflammatory responses are used to fuel infiltration of activated CD4<sup>+</sup> T cells and LCs at the vaginal mucosa<sup>198</sup>. Hence, HIV-1 exploits pDCs to overcome the problem of limited target availability at the mucosal sites of infection and thus favoring the systemic dissemination of the virus<sup>198</sup>. Importantly, the detrimental role of IFN $\alpha$  in the recruitment of target cells for systemic dissemination of the virus may only apply to the vaginal mucosa. Indeed, induction of antiviral mediators in the rectum, which contains many resident target CD4<sup>+</sup> T cells, might rather attenuate transmission<sup>200</sup>.

In addition, although BST-2 restricts transfer of virions from an infected cell to neighbouring cells by cell-to-cell spread, increased BST-2 expression on uninfected cells enhances their infection. The IFN-induced increase in BST-2 expression may thus benefit HIV-1 by enhancing cell-to-cell transmission<sup>158</sup>. Furthermore, the HIV-1 co-receptor CCR5 is upregulated via IFN $\alpha$  on T cell progenitors, which could contribute to the expansion of HIV-1 tropism *in vivo*. pDCs were shown to be able to transmit HIV-1 to mainly HIV-1-specific CD4<sup>+</sup> T cells. Conflicting results exist regarding the potential role of pDCs as reservoir for HIV-1. Otero et al. estimated that pDCs are not a major reservoir for HIV-1 *in vivo*<sup>201</sup>. In contrast, it has been suggested that pDC can be a HIV-1 DNA reservoirs in chronic infection with high levels of HIV-1 DNA load found in pDCs<sup>202</sup>. Taken together, these data suggest that IFN $\alpha$ /pDCs can contribute not only to viral spread during transmission but also may contribute to viral persistence.

#### **4.2.2 IFN $\alpha$ -mediated promotion of the loss of T cells in HIV-1 infection**

IFN $\alpha$  may contribute to CD4<sup>+</sup> T cell depletion in peripheral blood and secondary lymphoid tissues by several mechanisms. First, TLR7-induced IFN $\alpha$  can transform pDCs into killer pDCs<sup>203</sup>. TRAIL expression on pDCs was clearly demonstrated, including by microscopy analysis, on tonsils<sup>204</sup> and circulating pDCs<sup>48</sup> from viremic HIV-1-infected patients. TRAIL is induced on pDCs via IFNAR/STAT1 and STAT3 signaling while, simultaneously, the binding of HIV-1 to the CD4 receptor upregulates its ligand, death receptor 5 (DR5), leading to TRAIL-mediated apoptosis of DR5<sup>+</sup> cells, including HIV-1-infected CD4<sup>+</sup> T cells and uninfected bystander CD4<sup>+</sup> T cells<sup>146,205</sup>. TRAIL-mediated depletion of CD4<sup>+</sup> T cells may participate in the destruction of LN architecture in advanced stages of the disease<sup>147</sup>. Importantly, this mechanism may not be restricted to pDC-T cell interactions. TRAIL-mediated apoptosis of memory B-cell was reported in successfully treated HIV-1 patients<sup>206</sup>. TRAIL can also be induced by type I IFN on monocytes<sup>207</sup>. Excessive apoptosis induction in virus-infected cells downstream of high levels of type I IFN and TRAIL is emerging as a common pathway of immunopathology in virus infections as also reported in hepatitis C infection<sup>208</sup>.

Other mechanisms of apoptosis or cell death are also triggered in HIV-1 infection. IFN $\alpha$ -mediated upregulation of the pro-apoptotic molecules Bak and Fas may also participate in T cell depletion. *In vitro* treatment of T cells from HIV-1-infected patients with IFN $\alpha$  led to Fas-dependent apoptosis of T cells and Bak expression on CD4<sup>+</sup> T cells has been associated to CD4<sup>+</sup> T cell loss<sup>209</sup>. Binding of PDL1 to its receptor PD1 on T cells suppresses proliferation and can induce apoptosis. TLR7/8- or IFN-stimulated pDCs upregulate PDL-1<sup>210</sup> while PD-1 is upregulated in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HIV-1 infection. Type I IFN-regulated genes, including cell cycle-associated genes, are furthermore upregulated on activated CD4<sup>+</sup> T cells of HIV-1-infected patients<sup>12</sup>. IFN $\alpha$  may therefore promote T cell loss in HIV-1 infection by accelerating activation-induced cell death<sup>211</sup>.

#### **4.2.3. Role of IFN $\alpha$ in the non-specific T cell hyperactivation**

IFN $\alpha$  contribute to the upregulation of T cell activation markers and blocking IFNAR1 and IFNAR2 *in vitro* prevented the induction of the activation markers, CD38 and CD69, on HIV-1-exposed CD4<sup>+</sup> and CD8<sup>+</sup> T cells. *Ex vivo* T cell activation measure by CD38 expression is correlated to both plasma type I IFN activity and IFN $\alpha$  levels in HIV-1 infection<sup>185</sup>. IFN $\alpha$  administration significantly enhances CD8<sup>+</sup> T cell activation in chronically HIV-1-infected individuals. In addition, IFN $\alpha$  mediates the downregulation of molecules such as B and T lymphocyte attenuator (BTLA) on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Higher levels of IFN $\alpha$  and related inducible genes have been observed in lymphoid tissue derived from HIV-1-infected subjects with progressive disease as compared to uninfected controls<sup>12</sup>. The accumulation of mature pDCs in the LNs of HIV-1 infected individuals could therefore contribute significantly to generalized T cell activation. Besides, IFN $\alpha$  can inhibit telomerase activity and contribute to telomere shortening in human T cells<sup>12</sup>. The hyperproliferative state modulated by chronic IFN $\alpha$  secretion may drive memory T cell exhaustion in the long term<sup>12</sup>. Depletion of self-renewing central memory CD4<sup>+</sup> T cells by chronic stimulation in SIV infection has been shown to restrain the CD4<sup>+</sup> effector memory leading to immunodeficiency<sup>12</sup>. Overall, IFN $\alpha$  contributes to generalized T cell hyperactivation and associated impairments of T cell functions.

#### **4.2.4. Alteration of the balance between CD4<sup>+</sup> T cell subsets**

In addition to inducing T cell death and activation, the CD4<sup>+</sup> T cell subset population can also be modulated in HIV-1 infection. Two specific subsets of interest are Th17 cells, important for the preservation of the integrity of the gut and the prevention of bacterial translocation, and Tregs, potent suppressors of T cells and DC activation<sup>12</sup>. Tregs may also have an additional role in HIV-1 infection as they can simultaneously suppress HIV-1-specific T cell responses and the damaging immune activation<sup>12</sup>. The mechanisms of Treg/Th17 deregulation are not fully understood but may involve DC-derived cytokines and immunoregulatory enzymes. In particular, the IFN-inducible IDO can alter the Th17/Treg balance in favor of Treg induction through activation of the amino-acid starvation response gene EIF2AK4 in developing CD4<sup>+</sup> T cells, but also through the generation of

soluble tryptophan catabolites, which can contribute to Foxp3<sup>+</sup> Treg differentiation. The significance of this mechanism in untreated HIV-1 infection has been demonstrated by the ability of IDO1 activity to predict disease progression<sup>12</sup>. pDCs activated by HIV-1 and other TLR agonists can express IDO and regulate the interconversion of Th17 and Treg cells under inflammatory conditions<sup>12</sup>. In addition, Tregs can be induced by DCs with partially mature phenotype in LNs of HIV-1 infected patients<sup>212</sup>. Th17 loss contributes to further increase microbial translocation, therefore amplifying pathogenic immune activation and inflammation<sup>12</sup>. Chronic type I IFN production therefore induces a cascade of immune dysfunctions that only contribute to the devastating effect of HIV-1 infection on the host immunity.

## **5. Modulation of type I IFNs responses in HIV-1 infection**

### **5.1. Insights from specific models of infection**

Negative modulation of the immune response by persistent type I IFN in HIV-1 infection is not observed in all HIV-1 infected individuals and two infected population subsets are of particular interest.

#### **5.2.1. Lessons from HIV-2**

HIV-2 is a naturally occurring less pathogenic form of HIV infection and largely confined to West Africa<sup>213</sup>. HIV-1 and HIV-2 infected patients with comparable degrees of peripheral CD4<sup>+</sup> T cell depletion exhibit similar T cell activation<sup>213</sup> and reduction in circulating pDCs<sup>214</sup>. HIV-2 stimulation induced a gene expression pattern distinct from HIV-1, characterized by lower expression of type I IFN genes despite similar expression profiles of ISG, PRR and IFN-regulated viral restriction factors<sup>215</sup>. Importantly, HIV-2 stimulation favored pDC maturation into APC rather than into persistently IFN $\alpha$ -producing cells as in HIV-1 infection<sup>215</sup>. The favored APC phenotype observed in HIV-2 infection may promote efficient T cell responses resulting in prolonged immune-mediated control of viral replication in HIV-2 infection as compared to HIV-1 infection<sup>213</sup>. Differences in the intracellular trafficking of these two viruses leading to alternative maturation pathways could explain the different activated phenotypes of pDCs.

#### **5.2.2. Lessons from elite controllers**

A rare subset of individuals (<1%), called elite controllers (EC), are capable to suppress virus replication without treatment, preventing them from developing AIDS<sup>216</sup>. Importantly, HIV-1 infection in EC cannot be compared to benign infection of natural hosts given that EC do exhibit low viral production and abnormal immune activation and inflammation<sup>216</sup>. The level of T cell activation in EC is lower than in untreated viremic individuals, but higher than in healthy donors and individuals on successful ART<sup>216</sup>. *In vivo*, pDCs from EC do not express TRAIL on their membrane contrasting with the 30% of TRAIL-expressing pDCs observed in viremic

patients<sup>48,205</sup>. This was not an intrinsic defect, as pDCs from EC exhibit membrane TRAIL expression when stimulated *in vitro* by infectious or noninfectious HIV-1 particles, but rather linked to the low levels of virus found in EC. As highlighted in section III.4.2.2., TRAIL-mediated killing of bystander CD4<sup>+</sup> T cells contribute to IFN $\alpha$ -mediated HIV-1 immunopathogenesis. It is therefore tempting to hypothesize that T cell loss in chronic HIV-1 infection is reduced in EC due to the lower IFN $\alpha$  levels associated with a reduced number of HIV-1 particles. In spite of some described genetic factors related to protective HLA class I alleles<sup>216</sup>, the mechanisms underlying the natural control of HIV-1 in EC remain largely unclear. EC have smaller viral reservoirs compared with HAART-treated patients<sup>217</sup>. As viral reservoirs are established very early after HIV-1 infection, and in line with data from non-pathogenic SIV model, it can be hypothesized that innate immune responses during acute phase are central to determine the controller phenotype<sup>218</sup>. pDCs from EC are not only functional but appear to produce higher levels of IFN $\alpha$  in response to HIV-1 exposure than pDCs from viremic patients, which together with better CD4<sup>+</sup> T cell responses may lead to an efficient viral control<sup>218</sup>. Similarly to what is observed in the non-pathogenic SIV model, ISGs rapidly decline in EC following the acute phase of HIV-1 infection<sup>217</sup>. In addition, EC maintained elevated pDC blood number. It was recently shown that pDC frequency was one factor associated with viral load control in EC together with NK function and Pol-specific CD8<sup>+</sup> T cell responses<sup>219</sup>. The pDC contribution to elite control of HIV-1 may be related to positive effect of IFN $\alpha$  on both NK and CD8<sup>+</sup> T cell functions. Further research is needed to understand the role of pDCs/IFN $\alpha$  response in ECs.

## **5.2. Targeted modulation of type I IFN signaling *in vivo***

### **5.2.1. Therapeutic modulation of type I IFN response in acute HIV-1/SIV infection**

The potential benefit of early and transient administration of IFN $\alpha$  in acute HIV-1 infection has been evaluated by the group of Christine Rouzioux in the pilot Primoferon A study and the larger follow-up Interprim study<sup>220,221</sup>. Acutely infected patients were either treated or not treated with PegIFN $\alpha$ 2b during the first 14 weeks of HAART. Transient PegIFN $\alpha$ 2b prevented the rebound of HIV-1 replication at HAART interruption. Of note, IFN $\alpha$ 2b treatment stimulated anti-HIV-1 antibody production potentially through the increase of circulating levels of B cell-activating factor (BAFF). However, the higher concentrations of anti-HIV-1 antibodies in IFN $\alpha$ 2b-treated patients played little if any role in this limitation of viral replication rebound<sup>220</sup>. IFN $\alpha$  is more efficient for the viral control when administered in acute infection as compared to chronic infection in the context of HCV infection<sup>126</sup>, suggesting that IFN $\alpha$  administration early in viral infection facilitate viral control for a broad range of virus. Administration of type I IFN, once viral persistence has been established, may not be able to decrease viral titers<sup>222</sup>, however, it may be difficult to treat patients within the correct timeframe.

### **5.2.2. Administration of exogenous type IFN in chronically infected patients: Potential for HIV-1 control and eradication ?**

IFN $\alpha$  therapy was used early in the AIDS epidemic as one of the first anti-HIV-1 drug, both as

monotherapy or as a component of combination ART. But this therapeutic approach was abandoned due to the relatively modest antiviral effect and the toxicity associated with a high frequency of serious adverse events in HIV-1-infected individuals. IFN $\alpha$  therapy was used as the standard treatment in HCV infection in the 2000s<sup>126</sup>. The anti-HIV-1 properties of IFN $\alpha$  have therefore been examined in the context of large clinical trial investigating the effect of different IFN therapy on HCV infection in HIV-1-HCV co-infected individuals. Some studies reported a decline in HIV-1 VL upon PegIFN $\alpha$  treatment, including in the absence of concomitant ART<sup>223-226</sup>. But none of the studies were able to show full viral suppression by PegIFN $\alpha$ , which is achieved in 90% of the patients with modern ART regimens in the same short timeframe (12-24 weeks). Of note, all these studies were done in patients with a good immunological profile (CD4 count >400 cells/mm<sup>3</sup>), in line with restrictions at the time for introducing anti-HCV treatment in ART-naive HIV-1-infected patients with CD4 count <200 cells/mm<sup>3</sup>. One of the major concerns with the use of IFN $\alpha$  in the setting of HIV-1 infection is the possibility that it may lower CD4<sup>+</sup> T lymphocyte counts and further compromise immunity as described in section III.4.2.2. Interestingly, the fold induction of HIV-1 restriction factor expression in CD4<sup>+</sup> T cell correlated to HIV-1 viral load reduction during IFN $\alpha$ /ribavirin treatment in HIV-1-HCV co-infected patients<sup>226,227</sup>. Mathematical modeling of HIV-1 and HCV kinetics during PegIFN $\alpha$ /RBV treatment revealed that IFN $\alpha$  treatment affects HIV-1 and HCV differently. While it blocked HIV-1 *de novo* infection, it blocked HCV virions production from infected cells<sup>228</sup>. Importantly, induction of the interferon response pathway prior to initiation of IFN therapy in HIV-1 mono-infected patients was associated to the absence of effect on HIV-1 viral load<sup>225</sup>.

There is still a pronounced demand for alternative clinical management strategies due to frequent evolution of antiretroviral resistance, toxicity and the absence of a cure for HIV-1. The advantages of PegIFN $\alpha$  therapy over regular IFN $\alpha$  therapy, including higher stability and efficiency, have triggered a renewed interest for IFN therapy in HIV-1-infected individuals in the late 2000s. Angel and colleagues suggested that PegIFN $\alpha$  may be an option for HIV-1 treatment-experienced patients failing their current antiretroviral regimen<sup>229</sup>. Two recent studies have assessed the potential of IFN therapy in the maintenance of viral control during structured treatment interruptions (STI). Boué and colleagues found that PegIFN $\alpha$  did not prolong the time-to-treatment resumption, or even had deleterious effect in patients with a low nadir CD4<sup>+</sup> T cell count<sup>230</sup>. In opposite, Azzoni and colleagues reported a sustained control of viral replication in 45% of the 20 PegIFN $\alpha$ -2a-treated subjects after 12 weeks of ART cessation<sup>231</sup>. Immunomodulatory effects of IFN $\alpha$  were not assessed. Altogether, these studies do not support a rationale for IFN $\alpha$  treatment in chronically HIV-1 infected individuals, they highlight that type I IFN may still play a role in HIV-1 control.

### **5.2.3. Administration of type I IFN signaling inhibitors to enhance the immune reconstitution in HIV-1-infected patients receiving ART**

Pathophysiological parallels with patients with autoimmune disorders, such as SLE, the pathogenic role of IFN $\alpha$  has led to the idea that inhibition of type I IFN signaling in chronic HIV-1 infection may help reducing

persistent immune activation and subsequent disease progression<sup>66</sup>. Drugs inhibiting IFN $\alpha$  production such as hydroxychloroquine or humanized IgG1k monoclonal antibodies that inhibit the effects of IFN $\alpha$  in SLE (Sifalimumab and Rontalizumab) have been used successfully in SLE patients<sup>66</sup>. This rationale was supported by *in vitro* studies showing that decreased HIV-1-induced activation of pDCs through hydroxychloroquine or chloroquine-mediated inhibition of TLR7 signaling resulted in reduced expression of IDO and PDL-1 and reduced IFN $\alpha$  production<sup>66</sup>. Nevertheless, clinical trials on the use of hydroxychloroquine and chloroquine provided inconsistent results regarding T cell activation, viral loads and CD4<sup>+</sup> T cell recovery<sup>66</sup>. Blocking type I IFN signaling may foster secondary virus infections or enhance pathology of cytopathogenic viruses that can induce severe immunopathology<sup>126</sup>. While increased incidence have not been reported in phase I trials in autoimmune patients<sup>232</sup>, such secondary effects might be important for HIV-1 immunocompromised patients<sup>126</sup>. Altogether these data suggest that the repercussions of suppressing specific arms of the innate immune response are still unpredictable. Chloroquine and hydroxychloroquine may not be specific enough and more targeted strategies should be considered. Finally, the group of Daniel Douek has recently showed that transient antagonism of type I IFN signaling during the acute SIV infection lead to progression to AIDS<sup>233</sup>, arguing against therapeutic strategies to block type I IFNs signaling in acute infection. We will tackle the insights brought by this paper in more details in the discussion section. Improved therapeutic strategies can be designed based on the information provided by previous therapeutic interventions.

In conclusion, IFN $\alpha$  has a critical role in HIV-1 immunopathogenesis. Given that IFN $\alpha$  levels only partially normalize upon treatment, its deleterious effect likely persists even under suppressive ART. Furthermore, IFN $\alpha$ -driven inflammation and immune activation may be involved in the development of non-AIDS related events, which accounts for the shorter survival rate of HIV-1 infected individuals compared to healthy donors in the post-ART era. In the absence of therapeutic vaccines, strategies aiming at reducing IFN $\alpha$ -driven inflammation and immune activation are relevant. But further efforts are needed to identify new targets. Sex differences have been reported in the course of natural HIV-1 infection. We will now show how they articulate around immune activation and IFN $\alpha$ . Their study can identify new IFN $\alpha$  modulators but also provide critical information for better patient management.

## **V. Sex differences in IFN $\alpha$ and HIV-1 infection**

### **1. Mechanisms underlying sexual dimorphism in immune responses**

#### **1.1. Sexual dimorphism in immune responses**

Significant differences in immune responses between females and males have been long noted but often remain overlooked in immunological studies and particularly in studies of human viral infections<sup>234,235</sup>. Sex differences have been reported in the clinical manifestations, immune responses and outcome of viral diseases, including influenza A virus, hantavirus, HCV and HIV-1<sup>234-237</sup>. Enhanced responsiveness to vaccination in females can be counterbalanced by a greater prevalence of adverse events<sup>234</sup>. Furthermore, females are also often more prone to immune-mediated pathology and autoimmunity<sup>238</sup>. Females and males also age differently leading to different morbidity and mortality rates<sup>239</sup>. As a general rule, females exhibit more robust cell-mediated and humoral immune responses to antigenic challenges compared to males<sup>238</sup>. Females also exhibit more potent inflammatory responses<sup>238</sup>. Age- and sex-dependent alterations can be observed in the global gene expression profile of immune cells from young and elderly males and females<sup>240</sup>.

The identification of generalized mechanisms of sex differences in immune responses has been challenged by the under-representation of females in both animal and human studies. Women are under-enrolled in clinical trials<sup>234</sup>. While the inclusion of both male and female study subjects in animal studies should be the norm, it is regrettably not the case, largely because of the associated costs and the diminishing research funding. Sex bias in the immune responses could result from direct effects of an individual's sex chromosome complement (X- or Y-linked genes), from sex hormones, or from environmental risk factors that act in a sex-specific manner.

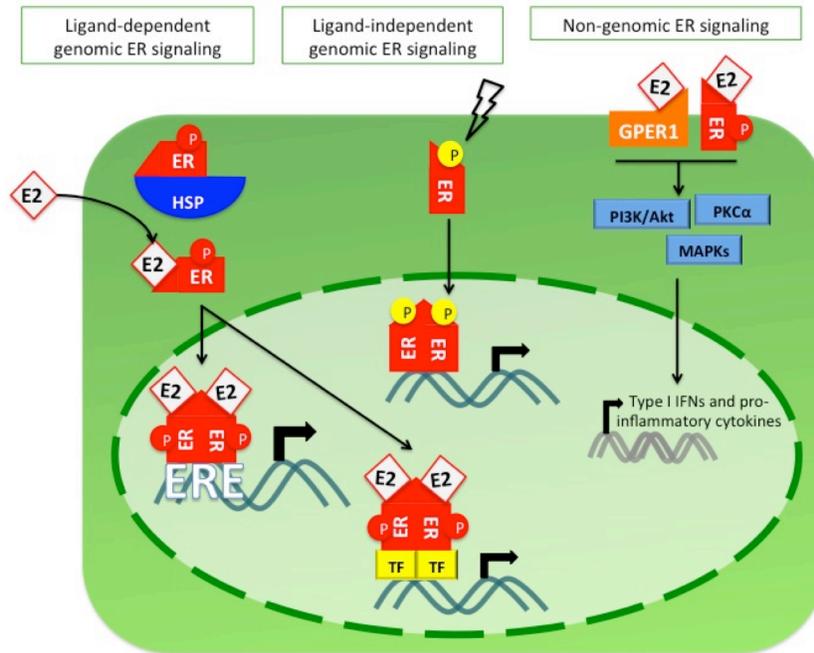
#### **1.2. Modulation of immune responses by estrogen signaling**

##### **1.2.1. Estrogens and their receptors**

17 $\beta$ -oestradiol (E2) is the active form of endogenous estrogens and the most abundant in females<sup>241</sup>. In addition to ovary cells, estrogens can be produced in extragonadal tissues such as adipose tissue or in the liver<sup>242</sup>. The levels of active steroid hormones in the circulation do not reflect their concentrations in tissues<sup>242</sup>.

The estrogen receptor (ER) $\alpha$  was discovered in 1960s while ER $\beta$  was only discovered in the mid-1990s<sup>242</sup>. ER $\alpha$  and ER $\beta$  are encoded by *Esr1* and *Esr2* respectively, which are located on different chromosomes. ERs are composed of five structural domains (A/B, C, D, E et F). Their DBD (C-domain) is conserved between ER $\alpha$  and ER $\beta$  and recognizes estrogen responsive elements (ERE) on DNA sequences<sup>241</sup>. ERs contain two transactivation domains responsible for the recruitment of coactivators such as the steroid receptor coactivator 1 (SRC-1),

HDACs, CBP/p300<sup>242</sup>: the NH2-terminal transactivation domain AF1 (A/B domain) and COOH-terminal



**Figure 12: Mechanisms of action of ERs**

The classical genomic ER signaling pathway depends on ligand binding and is mediated through nuclear ERs. Dimerized ERs can bind directly to DNA through ERE or indirectly via DNA-bound transcription factors (TF). Membrane ERs can be activated by E2 and triggers PI3K/Akt, PKC $\alpha$  and MAPKs signaling pathways. ERs can be phosphorylated at specific sites independently of ligand binding, leading to the transcription of target genes.

*Adapted from [251]*

transactivation domain AF2<sup>241</sup>. The AF2 domain contains the ligand-binding domain (LBD) (E/F domain)<sup>241</sup>. The role of AF1 and AF2 differ between ER $\alpha$  and ER $\beta$ <sup>243</sup>, cell types and target genes<sup>244-247</sup>. Shorter forms of ER $\alpha$  without AF1 are notably found in breast cancer cell lines where they inhibit transcriptional activity by forming heterodimers with complete isoforms<sup>241</sup>. ERs are nuclear receptors but certain ER $\alpha$  and ER $\beta$  variants are found on the plasma membrane<sup>241</sup>. ERs can be activated by different compounds such as isoflavonoides due to large enzymatic cavity<sup>248,249</sup>. Estrogens can also signal through G protein-coupled estrogen receptor 1 (GPER1), a plasma membrane receptor formerly known as an orphan G protein-coupled receptor 30 (GPR30).

Estrogens have a broad regulatory role in both males and females. Their pathological role has been extensively studied in the context of breast cancer while their protective role was studied in liver cancer<sup>250</sup>. Studies in murine models show that systemic exposure to tamoxifen, pharmacological ER ligand used in the treatment of breast cancers and osteoporosis<sup>251</sup>, modulates immune cell function<sup>252</sup>.

### 1.2.1. Mechanisms of action of ERs

ERs mediate effects on cell metabolism, cell differentiation, and tissue development through various

mechanisms as shown in Figure 12. In these processes, estrogen-targeted gene regulation generally requires the interaction between ER proteins and genomic DNA, in which ER acts as a transcription factor<sup>251</sup>. The classical genomic ER signaling pathway is mediated through nuclear ERs. In the absence of ligand, nuclear ERs are sequestered in the cytoplasm within a large inhibitory heat shock protein (HSP) complex. Binding of estrogen leads to rearrangement of the  $\alpha$  helices of ERs, enabling the displacement of heat shock proteins and facilitating the interaction of a receptor dimer within the regulatory regions of target genes. The interaction of ERs with target gene promoters can occur either directly, through specific estrogen response elements (EREs; 5'-GGTCANNNTGACC-3', N referring to any of A, C, G or T), or indirectly through contacts with other DNA bound transcription factors such as NF $\kappa$ B, SP1, Fos/Jun/AP-1, GATA binding protein 1 (GATA1) or STAT5<sup>242</sup>. In addition to regions that harbor half or degenerate EREs, which are also permissive to ER $\alpha$  recruitment, more than 70,000 EREs are scattered throughout the human genome, among which over 17,000 are within 15 kb of mRNA start sites<sup>253</sup>. Besides, around one third of the estrogen responsive genes lack ERE-like sequences<sup>242</sup>, highlighting the non-negligible contribution of indirect genomic ER signaling. ERs do not interact with the transcription apparatus in the same manner in all cells but ER $\alpha$  and ER $\beta$  mostly bind to the same binding sites<sup>254</sup>. Surprisingly, ER $\alpha$  binding does not occur at gene promoters but rather in regions  $>10^5$  bp distal from transcribed genes<sup>241</sup>. The state of chromatin compaction, whether open or closed, can directly impact DNA readability by ER $\alpha$ <sup>242</sup>.

Non-genomic ER signaling can be rapidly engaged by E2 through cell membrane ERs such as GPER1<sup>242</sup> and certain variants of ER $\alpha$  and ER $\beta$ . The phospholipase C and PKC $\alpha$ , the MAPK and PI3K/Akt pathways<sup>242</sup> have been shown to be activated by non-genomic ER signaling. As mentioned earlier, these pathways are implicated in the transcription of type I IFNs and pro-inflammatory cytokines downstream of TLRs.

Phosphorylation of ERs on certain residues or their associated co-regulators can cause ligand-independent ER activation<sup>242</sup>. Importantly, ligand-free and E2-bound ER $\alpha$  can oppose each other in transcription of the target genes as exemplified for *TNFA* gene in osteoblasts<sup>255</sup>. Altogether these data shows that the classic genomic mechanism contributes only a small part to the complexity of estrogen signaling, which can therefore modulate immune responses through multiple mechanisms.

### 1.2.3. Importance of estrogen signaling in immune responses

Estrogens control many aspects of our biology. The importance of sex hormones on immune responses was first highlighted in the context of autoimmune diseases by clinical evidences showing that menstrual cycle, pregnancy, and menopausal status, which are characterized by fluctuations of endogenous estrogens, significantly influence the course of autoimmune diseases. In particular, age-related changes in the female-to-male ratio were observed in SLE patients with a 3:1 ratio before puberty, a 9:1 ratio in child-bearing age followed by a decrease at menopause. It is generally acknowledged that E2 effect is biphasic<sup>252</sup>. Low levels of E2 stimulate Th1 responses such as TNF $\alpha$  while high levels of E2 are associated with Th2 responses such as IL-10 and IL-4<sup>252</sup>. At high levels, E2 inhibits important proinflammatory pathways including TNF $\alpha$ , IL-1 $\beta$  and IL-6. Of

note, the biphasic effect of E2 is not observed for B cells as antibody production is stimulated at both low and high concentrations of E2<sup>252</sup>. Interestingly, E2- and progesterone-mediated inhibition of cytokine production is greater in HIV-1-infected subjects (35% decrease for both hormones) compared with uninfected subjects (12% and 19% for estrogen and progesterone, respectively) whereas the effect on proliferation and PBMC phenotype did not differ by HIV-1 status<sup>256</sup>. This may be related to HIV-1-mediated gonadal dysfunction. E2 effect on cytokine production may be related to the ER-mediated modulation of NFκB signaling<sup>252</sup>. ER ligands physically regulate the activation and function of STAT3 and STAT5 in endothelial and epithelial cells as well as through the increased expression of inhibitors of cytokine signaling including SOCS2 and PIAS3. ER-mediated regulation of STATs signaling remains to be demonstrated in immune cells.

Estrogens can mediate their effects on immune responses via the regulation of miRNAs. For example, ERα-mediated estrogen signaling inhibits transcription of miR-21, while it stimulates expression of miR-203, among others<sup>242</sup>. miR-21 is a negative modulator of signal transduction downstream of TCR in T-lymphocytes and a major actor in the resolution of inflammation<sup>257</sup>. Some of the ER-regulated miRNAs have been reported to be dysregulated in autoimmune diseases including miR-21 and miR-203 in colonic tissue of patients with the inflammatory bowel disease and in synovial fibroblasts in patients with RA, respectively<sup>258</sup>. Last but not least, the estrogen-regulated miR-146a is recognized as a negative regulator of type I IFNs response by targeting TRAF6 and IRAK1<sup>71</sup>.

Different outcomes can be observed among different immune cells in response to the same hormone. The distinct expression patterns *in vivo* of the ERα and ERβ can bring a divergent signal<sup>252</sup>. ERβ may antagonize ERα-dependent transcription by altering the recruitment of regulatory factors or increasing ERα degradation<sup>251</sup>. ERs are expressed on all PBMCs including pDCs<sup>31,241,259,260</sup> but the relative expression of ERα and ERβ differ among cell types. *Esr1* mRNA expression is higher in B cells and T cells than in pDCs and monocytes while B cells and pDCs have higher levels of *Esr2* mRNA compared with T cells and monocytes<sup>31,260</sup>. Monocytes express low levels of both *Esr1* and *Esr2*<sup>31,260</sup>. Of note, no difference in *Esr1* expression levels in immune cells was observed between men and women with the exception of monocytes derived from men expressing more *Esr1* than monocytes derived from women<sup>31,260</sup>. The nature and extent of ER response to an estrogenic stimuli in a given cell type are determined by the proteins, pathways, and processes with which the receptor interacts. Not all cofactors are functionally equivalent, nor are they expressed in the same manner in all cells. Little is known about co-regulator expression in immune cells. Estrogen signaling can therefore orchestrate a global immune response resulting from distinct effect on various immune cells. Progesterone and testosterone can also influence immune responses. For instance, testosterone negatively regulates the production of TNFα, MIP-1α and MIP-1β<sup>261</sup>. The simultaneous presence of the different sex hormones may therefore further diversify the immune response. Importantly, immune responses or pathological state can also regulate ER signaling. Inflammatory state has been shown to upregulate expression of ERβ relative to ERα, therefore potentially favoring ERβ signaling pathways and altering subsequent estrogen effects<sup>252</sup>. In addition, ERα is directly

targeted by numerous miRNAs<sup>242</sup>.

Most studies on sex differences in viral infections have been and remain carried out in rodents<sup>240</sup>. The ways by which sex-dependent immune cell functions are influenced by sex hormones is an emerging field. While practically very challenging to implement, it is important to investigate mechanistically in humans the sex differences in immune responses as major differences exist between human and mice immune systems. Major open questions remain to be solved, including how does E2 influence proinflammatory cytokine secretion in the presence and absence of TLR stimuli and reciprocally how TLR stimulation may affect ER $\alpha$  and ER $\beta$  expression.

### **1.3. Modulation of immune responses by X-linked factors**

Females inherit two parental X-chromosomes. To avoid double expression, one gene from one of the parental X-chromosome is silenced during an early embryonic stage, a process referred as 'X-chromosome inactivation' (XCI). XCI is believed to occur randomly for each cell (i.e. there is a 50/50 ratio of the maternal and paternal X-chromosomes) and to be permanent for all descendants of that cell. Females possess therefore a mosaic of cells with either the maternal or the paternal X chromosome inactivated. It has been suggested that, uniquely for women, higher life expectancies could be the result of the mosaicism caused by XCI, as they have a choice between two cell populations. Nevertheless, defects in X-chromosome inactivation occur in about 10-15% X-linked genes<sup>262</sup>. Skewing of X chromosome inactivation may be primary, either due to chance or to factors that affect the process of XCI during early embryo development or secondary to a selection process against or in favor of cells with a specific genotype. It has been shown that XCI may be skewed in elderly individuals (older than 55–60 years) and continues to increase in populations at the most advanced ages. Interestingly, Mengel-Form and colleagues recently showed that this skewing is associated with survival<sup>263</sup> with women with a low degree of skewing (DS) having significantly higher mortality than the majority of women who had a more skewed DS. Understanding how this age-related skewing shapes immune responses in that context would be of great interest.

Several genes important for immune responses are found on the X-chromosome including TLR7, CD40L, IRAK1 FOXP3, which has been implicated in the development of regulatory T cells and receptors to IL-2, IL-3, IL-4, IL-9, IL-13 and IL-21 as well as genes encoding proteins involved in NF- $\kappa$ B signaling<sup>240</sup>. Besides, X chromosome is highly enriched for miRNAs, in contrast to the Y chromosome, which contains no miRNAs. Importantly, about 70% X-linked genes (with known function) are associated to diseases and may be particularly relevant to SLE<sup>240</sup>.

## **2. Sex differences in the natural course of HIV-1 infection**

In 1987, AIDS was the leading cause of death in women of reproductive age (15 to 44) in the USA. Thirty-three years later, 25-30% of all deaths in women between the age of 25 and 40 years old worldwide are still due

to AIDS and tuberculosis. Women represent 52% of all people living with HIV-1 in low- and middle-income countries while they represent a much lower proportion in high-income countries<sup>264</sup>. The majority of new HIV-1 infections in women in 2010 were diagnosed in women of child-bearing age (between 25 and 44 years of age). Several socio-economic factors render women more vulnerable to HIV-1 acquisition, especially in African nations and other resource-limited countries.

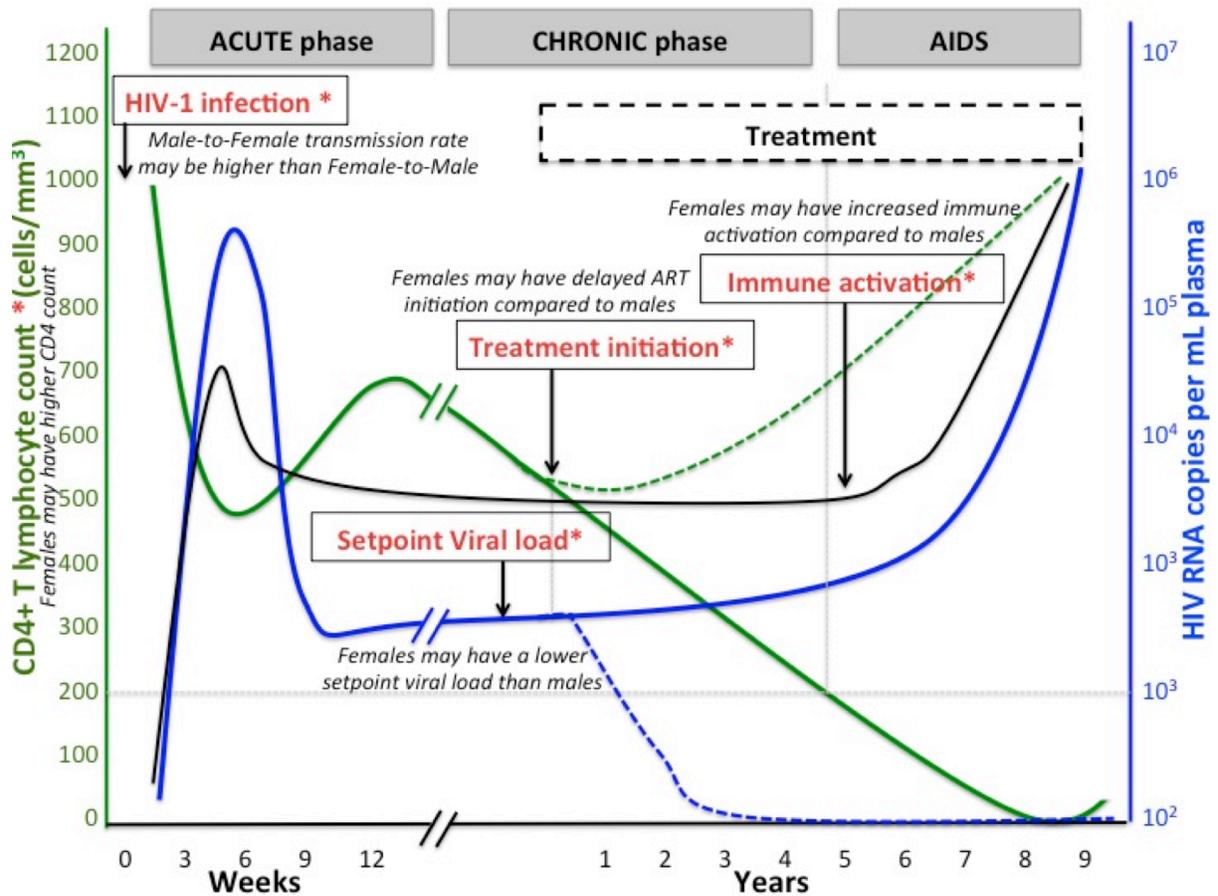
Sex differences in survival seem to mainly come from differential access to HIV-1 treatment given that among treated individuals, the survival did not differ by sex<sup>237</sup>. Higher mortality among men than women on ART in Africa has also been attributed to later presentation to ART programs by men<sup>265</sup>, suggesting that socio-economic factors are the main drivers of sex differences in mortality among HIV-1-infected individuals. However, there are increasingly data supporting a role for biological and physiological mechanisms underlying sex differences in the natural course of HIV-1 infection. We will now detail some of these aspects with sex differences throughout the course HIV-1 infection illustrated in Figure 13.

## **2.1. Sex differences in HIV-1 acquisition and transmission**

Heterosexual transmission was the major route of HIV-1 transmission in 2012 for both women and men, with 30-40% of annual HIV-1 infections worldwide occurring through heterosexual transmission in the female reproductive tract<sup>266</sup>. There is an increasing consensus that male-to-female transmission is more efficient than female-to-male transmission<sup>264</sup>. Several mechanisms may participate in women's biological greater susceptibility to HIV-1 heterosexual transmission. The frequency of Langerhans cells (LCs) in the mucosa epithelium, which have a crucial role in the early diffusion of the virus beyond the site of viral entry<sup>266</sup>, and the epithelium thickness differ between men and women but are influenced by sex hormones<sup>267</sup>. Men and women may also differ in the target cell availability. Importantly, sex differences in immune activation may be involved in women's greater susceptibility to HIV-1 heterosexual transmission. The analysis of the results from the CAPRISA 004 phase IIb, randomized, placebo-controlled clinical trial assessing the safety and effectiveness of 1% tenofovir gel in preventing HIV-1 infection in women, showed that women who acquired HIV-1 had significantly higher systemic innate immune activation prior to infection than women who remained uninfected, irrespective of microbicide use<sup>132</sup>. Studies on HIV-1-exposed seronegative (HESN) cohorts have also implicated elevated immune activation as a risk factor for acquiring HIV-1<sup>268</sup>. Reduced T cell activation is believed to limit the pool of activated CD4<sup>+</sup> T target cells permissive to HIV-1 infection and low levels of pro-inflammatory cytokines at the infection site reduces the recruitment of target cells to the site of infection. It is well established that systemic innate immune activation is in general higher in women than men<sup>240</sup>. Although a sex difference in immune activation at the genital mucosa has not been reported, it has been shown that women have higher gut mucosa immune activation than men<sup>269</sup>. Women might therefore be more susceptible to HIV-1 acquisition due to higher

numbers of activated target cells at the site of viral transmission resulting from increased immune activation and inflammation.

Sex differences in VL, discussed further below, might contribute significantly to sex differences in HIV-1 transmission <sup>270</sup>. While HIV-1 transmission from fully suppressed index cases is very low, it is important to highlight that HIV-1-infected individuals are particularly at risk of transmitting the virus during primary HIV-1 infection when viremia is high and HIV-1 status is frequently unknown. Therefore, even in the post-ART era, sex differences in VL may account for increased male-to-female transmission efficiency.



**Figure 13: Sex differences throughout the course HIV-1 infection**

Sex differences have been reported throughout the course of HIV-1 infection. Male-to-female transmission rate is higher than female-to-male transmission rate. Immunological and virological characteristics also appear to differ between the sexes with women generally having higher CD4+ T cell counts and lower viral set point than men. These differences may lead to delayed initiation of ART in women in regard to earlier guidelines. Women have also increased immune activation compared to men, which could impact the occurrence of AIDS- and non-AIDS-related events. Changes upon ART are shown with dotted lines.

## 2.2. Sex differences in Viral Load and Immunopathology

HIV-1 infection is characterized by a continuous decline in CD4<sup>+</sup> T cell counts, eventually leading to

immunodeficiency and AIDS. In general, HIV-1-negative women have higher CD4<sup>+</sup> T cell counts than HIV-1-negative men<sup>234</sup>. However, studies assessing sex differences in CD4<sup>+</sup> T cell counts have been discrepant<sup>271-274</sup>. Similarly, studies evaluating changes in CD4<sup>+</sup> T cell counts over time in women and men or CD4<sup>+</sup> T cell count recovery after initiation of ART have led to conflicting results. Greater T cell recovery in women than men was observed in developing countries, with sex-based differences increasing with time on ART. The factors responsible for these sex differences in CD4<sup>+</sup> T cell count are insufficiently understood, but might be related to differences in the level of immune activation in HIV-1-infected women and men.

HIV-1-infected women have significantly higher CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio than HIV-1-infected men due to lower CD8<sup>+</sup> T cell count among both HIV-1-infected and HIV-1-uninfected individuals<sup>275</sup>. The CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio has been shown to predict the risk of both AIDS and non-AIDS related morbidities with higher CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio associated with better prognosis<sup>276</sup>.

The majority of studies assessing sex differences in viral loads have shown lower viral loads in women compared to men despite some results arguing that there are no sex differences<sup>237</sup>. Several large studies have reported that women exhibit between 0.13-0.35 log<sub>10</sub> (about 50%) lower HIV-1 RNA levels early in the infection as compared to men<sup>236,275,277-283</sup><sup>274</sup>. These differences in viral loads persisted for several years after seroconversion<sup>274</sup> before attenuating, resulting in no detectable differences in viral loads at later stages of infection<sup>236,274,278</sup>. Furthermore, it is important to note that differences in HIV-1 RNA viral load between men and women have been shown to be larger for HIV-1-infected individuals with higher CD4<sup>+</sup> T cell counts<sup>275,284-288</sup>. Viral load suppression serves as an important clinical tool as it predicts the durability of responses to combination ART and decreases the risk for the development of drug resistance. Data relating to sex differences in virological responses to ART have been conflicting<sup>237</sup>. Possible mechanisms for improved viral suppression in women might be increased drug levels of antiretroviral drugs following standard doses in women particularly within the protease inhibitor class or lower pretreatment RNA as slower viral suppression has generally been associated with higher pre-ART HIV-1 RNA.

The mechanisms underlying these sex differences in VL, however, remain unknown. It has been postulated that the effect of sex hormones on chemokine receptor expression and cytokine production might influence viral replication and disease progression<sup>236</sup>. Notably, it has also been hypothesized that TNF $\alpha$ , which is associated with immune activation and increased viral replication, might be inhibited by estrogen, resulting in a lower viral load in women. However, no sex differences in TNF $\alpha$  production upon TLR7 stimulation, which is activated by HIV-1 ssRNA, have been demonstrated<sup>187,259,289</sup>. Finally, differences in IFN $\alpha$  production between women and men, as discussed further below, might have a direct effect on the observed sex differences in viral loads, as higher levels of IFN $\alpha$  production in women might lead to enhanced expression of IFN $\alpha$ -stimulated antiviral host restriction factors. A role of sex hormones is supported by the observation that the CD4<sup>+</sup> T cell count does not differ between men and women over the age of 50 and that viral load can vary during the menstrual cycle<sup>237</sup>. However, given that lower viral loads are also seen in girls compared to boys<sup>290</sup>, it appears

that sex hormones alone cannot account for all the sex differences in viremia and that more complex mechanisms, including X-chromosomally encoded genes, are most likely involved. Despite women having a lower viral load early in HIV-1 infection, similar rates of disease progression have been observed between men and women<sup>236,237,271,274,291-301</sup>, including after the introduction of ART in 1996, suggesting that the lower viral load observed in women does not lead to delayed disease progression.

### 3. Sex differences in the production of IFN $\alpha$

As described above, persistent production of type I IFNs by pDCs contribute to several aspects of HIV-1 immunopathogenesis. Importantly, in the context of sex differences in the manifestations of HIV-1 disease, it has been shown that pDCs derived from females produced markedly more IFN $\alpha$  in response to HIV-1-encoded TLR7/8 ligands than pDCs derived from males, resulting in stronger secondary activation of CD8<sup>+</sup> T cells<sup>187</sup>. Higher IFN $\alpha$  production by pDCs in response to TLR7 has also been observed for healthy women as compared to healthy men<sup>289</sup>. The observation of a trend towards a lower frequency of IFN $\alpha$ -producing pDCs in postmenopausal women compared women of childbearing age suggested a role for sex hormones. The precise functional mechanisms by which sex hormones might regulate the IFN $\alpha$  response of pDCs are unknown, but are thought to involve ER $\alpha$  signaling<sup>259,302</sup>. Short-term *in vitro* exposure to 17 $\beta$ -oestradiol (E2) did not affect the TLR-mediated responses of pDCs whereas *in vivo* treatment in post-menopausal women decreased the threshold of TLR responsiveness in human pDCs<sup>259</sup>, suggesting that the effect of E2 is independent of the presence of E2 at the time of TLR triggering *in vitro* but has an effect *in vivo*. Using a conditional mouse model, Seillet and colleagues further showed that the pDC IFN $\alpha$  response to TLR7 is positively regulated by E2 through estrogen receptor alpha (ER $\alpha$ ) during pDC lineage differentiation from progenitors<sup>259</sup>. The impact of E2 on IFN $\alpha$  production is pDC specific, as no difference in cytokine production by monocytes was observed<sup>259</sup>. In addition, X chromosome dosage may contribute, independently from sex hormones, to the sex bias in the pDC TLR7-mediated IFN $\alpha$  response<sup>31</sup>. *TLR7* is encoded on the X chromosome, however sex differences in pDC TLR7 response do not seem to be linked to higher expression of TLR7<sup>289</sup>. Altogether, these data suggest that the mechanisms underlying sex differences in IFN $\alpha$  production are very complex but also precise, as both timing and cell-specificity matter. Further research is needed to pinpoint targets for hormonal regulation.

It has been suggested that women have a higher risk of developing AIDS compared to men for the same level of viral replication<sup>236,284</sup>. Higher activation of T cells has been reported in women compared to men after controlling for viral replication, and was associated with increased IFN $\alpha$  production by pDCs<sup>187</sup>. Furthermore, increased levels of a subset of ISGs, including CCR5, MX-1 and ISG15, in CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells from treatment-naïve HIV-1-infected women have been observed during chronic HIV-1 infection after controlling for HIV-1 viral load<sup>162</sup>. The upregulation of those ISGs was associated with higher levels of immune activation in chronic HIV-1 infection<sup>162</sup>. Altogether, these data suggest that sex differences in the activity of TLRs might account for higher immune activation in women compared to men at a given HIV-1 VL and provide a mechanism

by which the same level of viral replication might result in faster HIV-1 disease progression in women<sup>187</sup>

Multiple studies have highlighted the crosstalk between sex hormones and immune responses in HIV-1 infection. IFN $\alpha$  and immune activation seem to be driving different aspects of sex differences in HIV-1 infection including lower VL and increased susceptibility to infection in females. The molecular mechanisms involved are only slowly starting to emerge. The role of X-chromosome linked genes remains largely unknown although their relevance has been highlighted by Siddiqui and colleagues. Indeed, they determined an association between an X chromosomal SNP and HIV-1 disease progression in women, but not in men<sup>303</sup>. In spite of major advances in the field, the critical relevance of sex to an immune response continues to be largely ignored. Creating a scientific culture that compels consideration of sex in shaping the immune response is essential to a comprehensive understanding of health and disease. Additional technical challenges will have to be faced to be able to fully consider sex as an integral component of an immune response.

## **V. HIV-1-HCV co-infection**

HIV-1 and HCV infections share important features, including route of transmission (bloodborne, sexual and vertical transmission), high mutability and rapid replication of the viruses as well as viral persistence. HIV-1 and HCV coexist in about 30% of HIV-1-infected individuals. In this section, we will highlight the impact of the IFN $\alpha$  and TLR7 pathways in HIV-1-HCV co-infection.

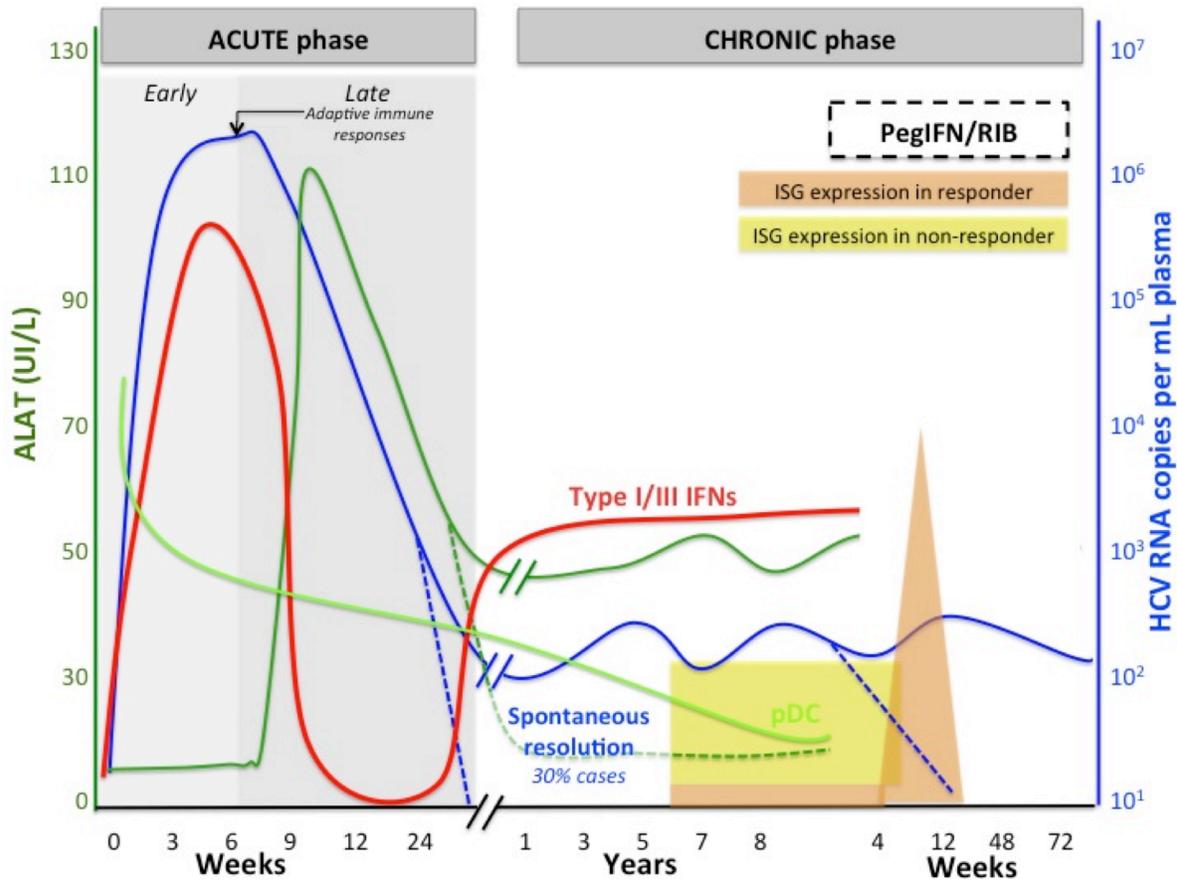
### **1. Natural course of HCV infection**

#### **1.1. HCV tropism**

HCV is a hepatotropic virus: its primary target cells are hepatocytes, highly specialized parenchymal liver cells. HCV internalization involves several host proteins, including the tetraspanin CD81 and the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN)<sup>304</sup>. A recent study on human liver biopsies from HCV infected individuals revealed that infected hepatocytes occurred in clusters, pointing to cell-to-cell spread as the predominant mode of HCV transmission<sup>305</sup>. Of note, in patients with high VL, the proportion of infected hepatocytes is in the range of 20%-50%<sup>305</sup>.

Several studies have reported detection of HCV in PBMCs including B lymphocytes, monocytes/macrophages and DCs<sup>306</sup>. Whether or not HCV has a preferential tropism for specific PBMCs subsets is still under debate. HCV lymphotropism may be of biological significance. Infection of PBMCs by HCV may correlate with the degree of liver damage<sup>306</sup> and contribute to the recurrence of HCV infection and difficult-to-treat disease status<sup>306</sup>. Besides, HCV infection of PBMCs may disturb immune responses<sup>306</sup>. Interestingly, HCV RNA is more frequently observed in PBMCs and T lymphocytes from HIV-1-HCV co-infected individuals than HCV mono-infected individuals, which may be related to increased cellular activation<sup>306</sup>. HCV is classified in seven genotypes that differ by about 20-30% at the amino acid level and multiple subtypes for each genotype<sup>307</sup>. Genotype 1 is the most prevalent (46%), followed by genotype 3 (30%), genotypes 2, 4 and 6 (cumulatively approximately 22%) and genotype 5 (less than 1%)<sup>308</sup>. HCV genotype 1 strains may have distinct lymphotropisms compared to HCV non-genotype 1 strains<sup>306</sup>. HCV genotype may represent a major confounding factor in the analysis of HIV-1-HCV co-infection and the immune response.

#### **1.2. Immune dysfunction in chronic HCV infection**



**Figure 14: Plasma levels of IFN $\alpha$ , peripheral pDCs numbers and clinical parameters during HCV infection**

HCV viral load and plasma type I/III IFNs levels peak in early acute infection. The late phase of acute infection is characterized by the initiation of adaptive immune responses, resulting in the decrease of HCV VL and IFN levels. Spontaneous resolution occurs in 30% of cases. ALAT levels are elevated in chronic infection, illustrating liver damage. Peripheral pDCs numbers are decreased during HCV infection. PegIFN/RIB is associated with reduction of HCV VL in responders. Responders to PegIFN/RIB treatment exhibit low levels of ISGs pre-treatment while non-responders have high levels of ISGs. Adapted from [310]

Acute HCV infection spontaneously resolves in 20-40% individuals. In 70–80% of those infected, the virus persists and the infection becomes chronic<sup>309</sup>. Chronic HCV infection is characterized by hepatic infiltration of immune cells<sup>310</sup>. Accordingly, high levels of chemokines including CXCL9, CXCL10, CXCL11, CCL3, CCL4, and CCL5 are found in the blood of HCV-infected patients<sup>311,312</sup>. While systemic immune dysfunction in HCV infection is controversial, it is generally acknowledged that immune dysfunction is not equivalent to what is seen in HIV-1 infection. No difference in CD8<sup>+</sup> T cell activation between HCV mono-infected individuals and healthy donors have been observed<sup>313,314</sup>. Defective responses to general PRR stimulation including decreased IFN $\alpha$  and IL-12 secretion, reduced CD86 expression, decreased HLA-DR and impaired stimulation of T cells in mixed lymphocyte reaction compared with normal controls have also been noticed in chronically HCV infected patients<sup>315-319</sup>.

Most patients develop some degree of liver fibrosis, and in 15–25% of patients cirrhosis occurs after 10 to 40 years<sup>309</sup>, which can be caused by damage to neighboring tissue from persistent yet ineffective inflammatory responses. Importantly, chronic HCV infection is characterized by a strong and persistent upregulation of ISGs in the liver. IFN $\lambda$ 1-4 mRNA but not IFN $\alpha/\beta$ , can be detected in liver biopsies. But no IFN proteins have been detected so far in liver biopsies of patients with chronically HCV-infected patients<sup>309</sup>. Questions remain opened concerning the factors that determine the activation level of the IFN system. Figure 14 shows the evolution of clinical parameters and IFN pathway in HCV infection. We will now discuss innate sensing of HCV and subsequent IFN induction.

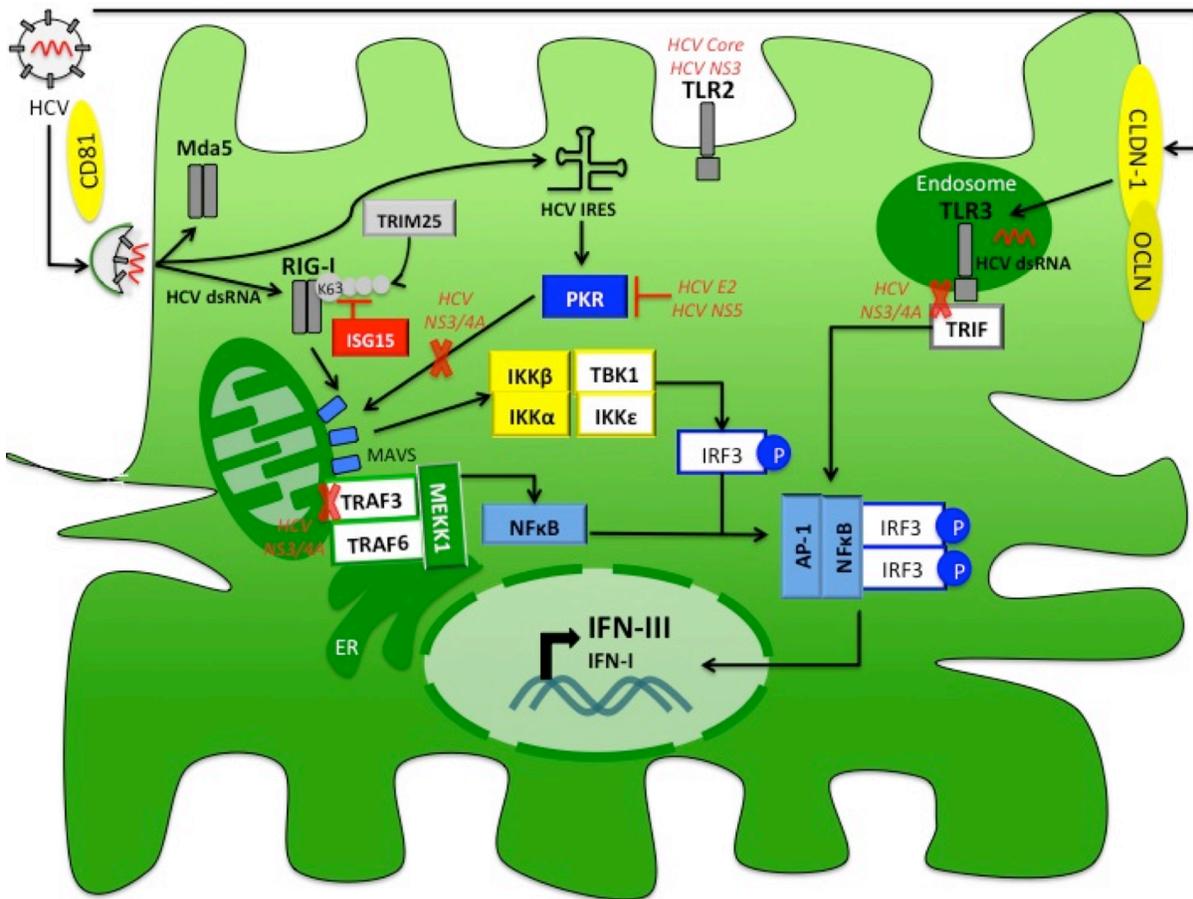
## 2. HCV sensing and IFN induction

### 2.1. Innate sensing of HCV

HCV is sensed in hepatocytes through the RIG-I and TLR3 pathways both of which recognize HCV dsRNA<sup>320-324</sup>. Figure 15 summarizes the molecular mechanisms underlying HCV sensing in hepatocytes. RIG-I recognizes short HCV dsRNAs with 5'-triphosphates within hours of infection<sup>304</sup>. Without viral stimulation, RIG-I is in an auto-inhibitory state. Binding to dsRNA induces conformational changes in RIG-I that facilitate its oligomerization and translocation from the cytosol to the mitochondrial-associated membrane (MAM)<sup>304</sup>. TRIM25 mediates K63-linked ubiquitination of RIG-I which necessary for RIG-I binding to its downstream effector mitochondrial antiviral signaling protein (MAVS) and for subsequent IFN production<sup>304</sup>. The interaction between RIG-I and MAVS promotes the formation of a signaling complex on the mitochondrial surface involving IKK $\alpha$ , IKK $\beta$ , TBK1 and IKK $\epsilon$  which activates IRF3<sup>304</sup>. RIG-I stimulation pathway also involves TRAF3, TRAF6 and mitogen-activator protein kinase/extracellular signal-regulated kinase (ERK) kinase 1 (MEKK1) which activates NF- $\kappa$ B<sup>304</sup>. RIG-I expression is notably enhanced by IFN. HCV has evolved mechanisms to counteract RIG-I function. Once sufficient viral proteins have accumulated in the cytosol, HCV NS3/4A protease targets the MAM-anchored synapse, cleaving MAVS from the MAM (but not from the mitochondria) and ablating RIG-I-mediated innate immune signaling. Similarly, NS3/4A proteins degrade TIR-domain-containing adapter-inducing IFN $\beta$  (TRIF), thereby interfering with IRF3 and NF- $\kappa$ B activation and subsequent TRIF-dependent IFN $\beta$  production<sup>304</sup>. Using the HCV-Huh7.25.CD81 infection system, Arnaud et al. dissected the acute IFN response to HCV infection into early, PKR, and late, RIG-I, phases<sup>325</sup>. HCV infection rapidly induces the expression of many IRF3-dependent genes, including ISG15, through a PKR-dependent mechanism before the RIG-I phase, which recruits MAVS<sup>325</sup>. Then, ISG15 induction blocks HCV RNA-mediated RIG-I activation by inhibiting RIG-I ubiquitination, thereby negatively controlling the RIG-I/MAVS pathway<sup>325</sup>. ISG15/ISGylation was shown to facilitate HCV production in the genotype 2a J6/Japanese fulminant hepatitis-1 (JFH-1) chimeric HCV infectious

model<sup>304</sup>. Type III rather than type I IFNs are primarily induced in hepatocytes in response to *in vitro* stimulation by HCV<sup>326</sup>.

Primary cultured hepatocytes express TLR1–9, and can respond to TLR2 and TLR4 ligands<sup>327</sup>. HCV Core and NS3 proteins can be detected by TLR2<sup>327</sup>. Recently, it was shown that HCV can be recognized by Mda-5 in primary hepatocytes<sup>328</sup>. Hepatocytes account for 60–80% of the total cell population in the liver while non-parenchymal cells consist of Kupffer cells (KC), DCs, lymphocytes, hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSEC) and biliary cells<sup>327</sup>. Each cell population exhibits different TLR expression<sup>327</sup>. KC express TLR2, TLR3, and TLR9 and respond to their ligands, hepatic NK cells express TLRs1, 2, 3, 4, 6, 7, 8, and 9 and respond to the corresponding TLR ligands, and HSCs express all TLRs<sup>327</sup>. While recombinant HCV core protein is



**Figure 15: Innate sensing of HCV in hepatocytes**

HCV dsRNA can be sensed in hepatocytes through RIG-I and TLR3. Upon activation, RIG-I associates with MAVS, triggering signaling pathways resulting to NF-κB and IRF3 activation and subsequent type I and III IFNs production. In addition, HCV IRES can be recognized by PKR. HCV can also be sensed by Mda5 and TLR2. However, HCV proteins can block the major sensing pathways in hepatocytes.

*Adapted from [324]*

not able to activate human HSCs, the latter respond to HCV replicon RNA via TLR3 by antiviral cytokines<sup>329</sup>. Liver sinusoidal endothelial cells respond to TLR1, 2, 4, 6, 9 ligands by producing TNF $\alpha$ , and respond to TLR3 ligands by producing TNF $\alpha$ , IL-6, and IFN $\beta$ <sup>327</sup>. TLR3 or TLR4 activation by polyIC and LPS respectively in murine KC and LSECs induce IFN $\beta$  which mediates antiviral activity against HCV<sup>330</sup>. HCV core and NS3 proteins can trigger TLR1, TLR2, and TLR6 signaling on monocytes to enhance the production of inflammatory cytokines<sup>327</sup>. HCV sensing in the liver, therefore, may take place in different cell types with varying responses.

## 2.2. pDC sensing of HCV and IFN $\alpha$ production

As expected, ssRNA HCV activates TLR7<sup>319</sup>. In addition to pDCs, TLR7 is also expressed in uninfected and HCV-infected hepatocytes<sup>319</sup>. TLR7 stimulation has been shown to reduce HCV mRNA and protein levels in isolated Huh-7 hepatocytes not only by stimulation of IFN production but also by direct activation of antiviral mechanisms in hepatocytes<sup>319</sup>. HCV virions are poor stimulants for pDCs TLR7 and TLR9<sup>331,332</sup>. IFN $\alpha$  production by pDCs requires cell-to-cell contact and is proportional to the number of HCV-infected hepatocytes<sup>333</sup>. Recognition of HCV-infected hepatoma cells by pDCs involves CD81<sup>334</sup> and its binding partner CD9 expressed on pDCs and not on HCV-infected cells, and Rac activity<sup>335</sup>. HCV RNA may not trigger IFN $\alpha$  systematically in pDCs as HCV RNA can be detected in 8% of IFN $\alpha$ -negative pDCs<sup>336</sup>. While it is possible that this observation may be linked to kinetics with threshold-dependent IFN $\alpha$  production- at least 45 HCV-RNA copies per pDC are needed for robust pDC activation<sup>336</sup>- one can also consider that differential responsiveness to HCV RNA stimulus may be related to heterogeneity among the pDCs population. Irrespectively, this observation suggests that HCV-mediated stimulation of IFN $\alpha$  production in pDCs is regulated by complex mechanisms. HCV subgenomic replicon cells, that replicate the viral RNA but cannot produce infectious virus particles, can also trigger pDCs to produce type I IFN, suggesting that induction of the IFN response by pDCs is independent of virus production<sup>333</sup>. Within pDCs, cell-culture-adapted strains of HCV (HCVcc) localized in organelles expressing the transferrin receptor CD71 but not Rab5 or LAMP-1, indicating that HCVcc antigen accumulates in recycling endosomes<sup>337</sup>. It has been hypothesized that HCV-derived antigens are targeted to recycling endosomal compartments to ensure a rapid activation of the HCV-specific CD8<sup>+</sup> T cell response by utilizing a storage pool of MHC class molecules<sup>337</sup>. Dreux et al. showed that inhibitors of exosome production ablated stimulation of pDC by HCV, therefore highlighting an unappreciated role of exosomes as PAMP carriers<sup>336</sup>. pDCs can also be stimulated by HCV-infected cells in a paracrine fashion by IFN $\alpha$ <sup>333,336</sup>. Grabski and colleagues recently demonstrated in an elegant study that efficient virus assembly, but not infectivity, determines the magnitude of HCV induced IFN $\alpha$  response by pDCs<sup>338</sup>. They first observed surprising differences in the IFN $\alpha$  induction capacity of two cell-cultured derived genotype 2a viruses. JFH-1 did not lead to IFN $\alpha$  production by pDCs. In contrast, insertion of J6CF derived core sequences into a JFH1 backbone significantly increased the induction of IFN $\alpha$  and was associated to increase

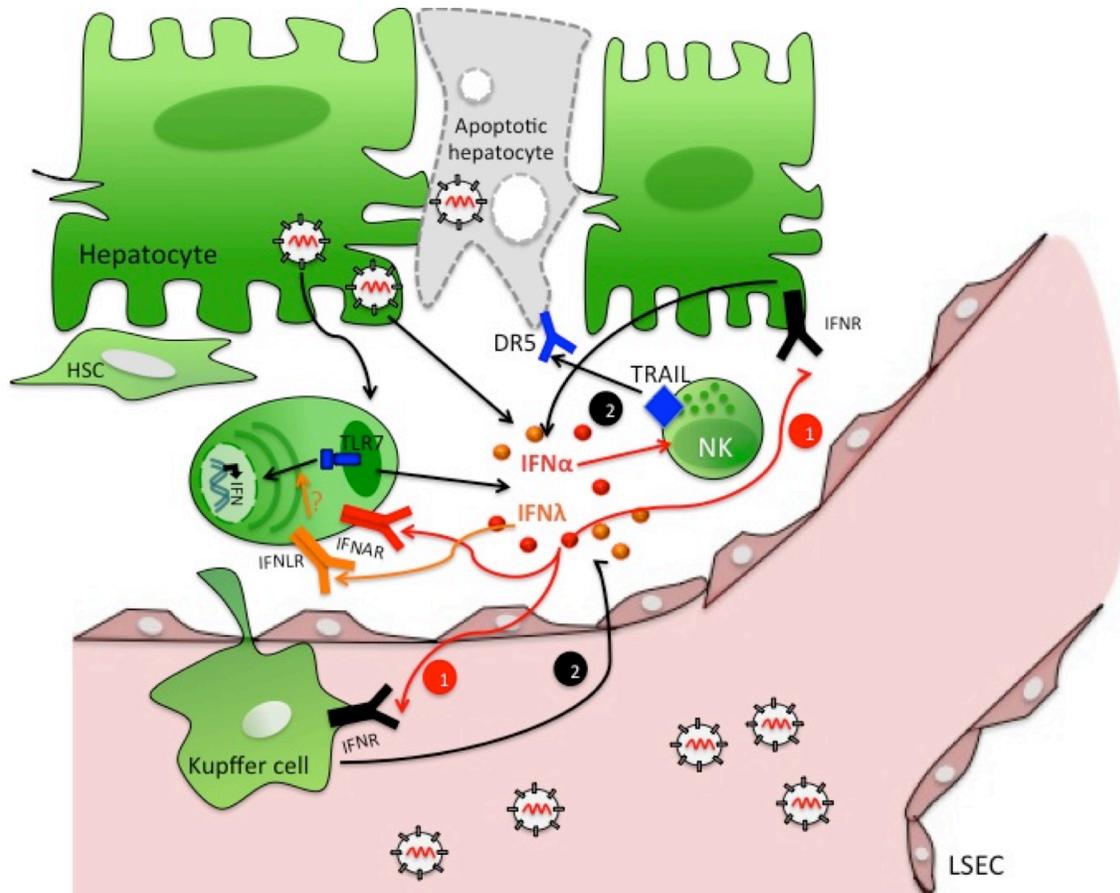
viral assembly<sup>338</sup>. Further proof of the critical role of virus assembly in the determination of the magnitude of interferon responses by pDCs comes from the use of an assembly deficient NS5AΔD3 mutant, which did not trigger pDC-derived IFN $\alpha$  production. The completion of membrane envelopment was needed to fully activate pDC<sup>338</sup>. HCV NS5 is also capable of impairing pDC function and decreasing pDC levels in patients with chronic HCV infection<sup>339</sup>. HCV Core interaction with IL-10 reduces IFN $\alpha$  production in pDCs, and may be crucial in HCV infection<sup>340-342</sup>. HCV can therefore modify TLR7 associated pDCs IFN responses to enhance virus survival.

RIG-I signaling has been described as largely dispensable for pDC secretion of IFN $\alpha$  following infection with RNA viruses<sup>343</sup>. However, Stone and colleagues recently showed that the Gen2.2 pDC-like cell line and human primary pDCs can respond to HCV PAMP through RIG-I, which is upregulated in response to HCV RNA<sup>344</sup>. Viral transcription is not required for RIG-I activation. Of note, the pDC-GEN2.2 cell line exhibit AA genotype for the RIG-I SNP (rs10813831)<sup>344</sup>, which results in an amino acid change (Arg to Cys) associated with increased expression of RIG-I and IFN $\beta$ 1. In addition to RIG-I as an alternate pathway for IFN production, the cellular source of type I IFNs in early acute phase of HCV remains under debate. Primary human hepatocytes (PHH) infected with HCV<sup>326,345,346</sup> and pDCs<sup>333</sup> stimulated by cell-cell contact with infected hepatocytes, have both been proposed as main producers of type I IFNs. Production of type I IFN in HCV infection can therefore be the result of various pathways and cell populations and these factors need to be carefully considered in the setting of HIV-1-HCV co-infection.

### **2.3. Importance of TLR7 signaling**

Genetic polymorphisms in TLR7 have been associated with differential liver fibrosis progression<sup>319</sup>. This effect may be linked to differential production of the anti-fibrotic cytokine IL-6 as suggested by the correlation between the protective variant c.1-120T > G in terms of fibrosis and inflammation and increased secretion of IL-6 in male patients<sup>319</sup>. In addition, impaired TLR3 and TLR7/8 function was reported to affect rapid fibrosis progression post-liver transplantation in HCV infection<sup>347</sup>. TLR7 polymorphisms are associated with response to IFN-based therapy in chronic HCV patients, with a gender-specific effect being observed in patients of Caucasian and Chinese descents<sup>319</sup>. Recently, the TLR7 rs3853839C allele, which is associated with a decrease in TLR7 transcripts, impaired IFN $\alpha$  production, and relatively more pronounced antiviral IL-6 production, has been suggested to confer a protective effect against HCV persistence in Chinese females<sup>348</sup>. It has been hypothesized that weakened TLR7 mRNA expression of this variant may be linked to differential miRNA binding activity<sup>348</sup>. However, HCV virological non-responders to IFN $\alpha$  therapy have been shown to exhibit reduced levels of TLR7 expression compared to virological responders<sup>349</sup>. In contrast, clinical trials with TLR7 and TLR9 agonists reduced HCV viremia in chronic infection<sup>319</sup>. As such the benefit/damage of TLR7 signaling in HCV infection is not entirely clear and may depend on the HCV subtype.

## 2.4. Crosstalk between type I and type III IFNs in HCV infection



**Figure 16 : Crosstalk between IFN $\alpha$  and IFN $\lambda$  signaling in HCV-infected liver**

IFN $\alpha$  and IFN $\lambda$  are initially produced by infected hepatocytes and pDCs. They are activating uninfected hepatocytes and Kupffer cells, leading to more IFN production. The IFN $\alpha$  produced upregulates TRAIL on NK cells, allowing them to kill infected hepatocytes, therefore contributing to liver damage. The effect of IFN $\lambda$  signaling on pDC IFN $\alpha$  production remains unclear.

Adapted from [324]

Type III IFNs consist of four members, termed IFN- $\lambda$ 1 (IL-29), IFN- $\lambda$ 2 (IL-28A), IFN- $\lambda$ 3 (IL-28B) and the recently identified IFN- $\lambda$ 4. The regulation of *IFNL1* gene was initially suggested to be similar to *IFNB* gene, relying on virus-activated IRF3 and IRF7 whereas *IFNL2/3* gene expression is mainly controlled by IRF7, similarly to IFN $\alpha$ , and seen to have delayed expression kinetics<sup>350,351</sup>. Subsequent studies highlighted key differences in the regulation of *IFNL1* and *IFNB* genes including IRF-independent regulation of *IFNL1*. Importantly, type III IFN induction may depend on peroxisomes-associated MAVS, which has been shown to induce ISGs expression but not type I IFNs upon RIG-I-like receptor (RLR) signaling. Type III and type I IFNs have been shown to upregulate distinct subsets of ISGs with different kinetics of induction<sup>343</sup>. Type III IFNs induce a more prolonged ISG induction in primary hepatocytes than type I IFNs but the induction of antiviral genes such as Mx2 was stronger for type I IFNs<sup>326</sup>. Type III IFNs have a decreased potency in regard to STAT1 activation compared to IFN $\alpha$ <sup>352,353</sup>.

They can be induced by type I IFNs<sup>350</sup> and can inhibit of HCV replication<sup>353,354</sup>. Type III IFNs activity is restricted compared to type I IFNs. Indeed, the IFN $\lambda$  receptor is not expressed ubiquitously but mainly restricted to epithelial cells<sup>343</sup> and human hepatocytes<sup>354</sup>. Immune cells are largely unresponsive to type III IFN except monocyte-derived macrophages and DCs with pDCs being responsive to IFN $\lambda$ s<sup>355</sup>. *IFNL3* is upregulated in hepatocytes and PBMCs from HCV infected individuals. In parallel, TLR7-stimulated pDCs can also produce large amounts of IFN $\lambda$ 3<sup>356</sup>.

The impact of type III IFNs on pDC IFN $\alpha$  response is conflicting. It was reported that IFN $\lambda$ 3-stimulated pDCs have increased production of IFN $\alpha$ <sup>357</sup> and that IFN $\lambda$ s may act as an autocrine signal for pDCs with the ability to improve survival and enhance antiviral response<sup>358</sup>. However, Egli et al. have investigated the crosstalk between type I and type III IFNs in the context of CMV infection. They showed that IFN $\lambda$  pretreatment of PBMCs resulted in lower IFN $\alpha$ 2 signaling and IFN $\alpha$ -induced ISGs induction<sup>359</sup>, suggesting that IFN $\lambda$  may rather inhibit type I IFN signaling. In addition, type III IFNs dictate response to IFN $\alpha$  therapy as discussed below. Hence, it remains to understand whether or not co-expression of IFN $\alpha$  with IFN $\lambda$  may represent two components of a coordinate response by multiple cell types<sup>360</sup>, in which the restricted induction of IFN $\lambda$  by virally infected non-hematopoietic cells amplifies, modifies and prolongs the effects of type I IFNs<sup>360,361</sup>. Figure 16 highlights how type I and III IFNs interact in HCV-infected liver.

## 2.5. Mechanisms of HCV interference with the type I IFN response

HCV-encoded viral protein interfere with various pathways regulating type I IFN expression. First, HCV may directly affect the expression of PRRs. Indeed, PBMCs from chronically HCV-infected patients expressed increased levels TLR2, TLR3, TLR4, TLR6 and TLR7<sup>362,363</sup>. NS5 inhibits the recruitment of IRAK1, resulting in a decrease in TLRs-MyD88-dependent signals<sup>304</sup>. NS5A expression is sufficient to rescue the replication of an IFN-sensitive virus. HCV inhibits basal level expression of IRF7 at both mRNA and protein levels and NS3/4A protease inhibits IRF7-induced IFN $\alpha$  promoter activation<sup>304</sup>. IRF7 may play an important role in eliminating HCV infection as suggested by the increased susceptibility of vHPV18/E6E7-immortalized hepatocytes to HCV infection upon IRF7 silencing<sup>304</sup>. In addition to direct role on downstream molecules of PRR signaling and PRR expression, HCV may also affect IFN expression through the modulation of negative feedback loops. HCV core protein has been shown to induce SOCS3 and suppresses IFN-mediated ISG expression<sup>304</sup>. In addition, SOCS3 expression is upregulated in chronically HCV-infected patients who are IFN non-responders compared with responders<sup>364</sup>. NS5A increases expression of IL-8, which in turn, inhibits IFN anti-viral activity and facilitates virus infection<sup>304</sup>. Importantly, the serum level of IL-8 is elevated in chronic hepatitis C patients compared with control individuals or is higher in IFN non-responders relative to responders. miR-21 is upregulated in liver samples from hepatocellular carcinoma patients and in HCV-infected cells<sup>304</sup>. It suppresses the expression of MyD88 and IRAK1, thereby repressing the production of type I IFNs<sup>304</sup>. HCV therefore also uses miRNAs to hijack

IFN responses. Plasma levels of IFN $\alpha$ 2 may be higher in HIV-1-HCV co-infected individuals than in HIV-1-mono-infected individuals<sup>365</sup>, although the results of this study should be very carefully considered given the low number of patients studied. Nevertheless, this suggests that in the context of co-infection with HIV-1, HCV may not be able to properly hijack IFN responses.

In addition to altering the IFN response, HCV can specifically suppress the induction of ISGs by multiple mechanisms. Core protein inhibits IRF1 synthesis, transcriptionally repressing several ISGs, such as IL-15, IL-12 and PKR<sup>304</sup>. HCV decreases methylation of STAT1, through the protein phosphatase 2A (PP2A)-mediated inhibition of the enzymatic activity of protein arginine methyl-transferase 1 (PRMT1), resulting in impaired nuclear translocation into the nucleus, binding of STAT1 to the ISRE, and ISG production<sup>364</sup>. HCV-mediated PP2A upregulation also enhances NS3 helicase activity by inhibiting PRMT1 enzymatic activity, which in turn facilitates virus replication. Interestingly, HCV-mediated suppression of IFN response occurs even before completion of HCV replication cycle. HCV polyprotein expression in replicon cell culture models is associated with a decrease in the expression of OAS and MxA and STAT1. STAT1 and STAT3 proteins are reduced in all major immune cell populations and hepatocytes from HCV-infected patients and STAT3 is preferentially ubiquitinated and targeted for proteosomal degradation in the presence of HCV<sup>366</sup>. In spite of the aforementioned effects of HCV on ISGs transcription, hundreds of ISGs are upregulated in patients with chronic HCV infection in both the liver and peripheral blood. Increased ISG expression are found both in HCV-infected hepatocytes and the neighboring uninfected hepatic cells from chronically infected patients, suggesting that HCV prevents neither the induction of IFN nor signal transduction through the Jak-STAT pathway in the liver of HCV-infected patients<sup>305</sup>. It has been suggested that HCV may also inhibit the translation of ISGs<sup>367</sup>. HCV has been shown to activate PKR, which phosphorylates eukaryotic translation initiation factor (eIF)2 $\alpha$ , resulting in the inhibition of cap-dependent protein translation<sup>324</sup>. Of note, HCV protein production is not impaired, because HCV RNA translation occurs through an internal ribosomal entry site (IRES) dependent mechanism that is not impaired by phosphorylated eIF2 $\alpha$ <sup>324</sup>. It is controversial whether the inhibited ISGs exhibit anti-HCV activity. It was reported that the upregulated expression of ISGs, such as OASL, ISG15 and viperin, in liver biopsies from HCV-infected patients and infected hepatocytes was correlated to decreased viral RNA levels. Nevertheless, the majority of studies tend to agree that viral replication is not efficiently restricted by the upregulation of hundreds of ISGs in patients with chronic HCV infection<sup>367,368</sup>. It remains unclear why the endogenously induced expression of ISGs is ineffective against HCV, because the same set of genes is highly effective when induced in non-preactivated patients during treatment with pegylated IFN (pegIFN) and ribavirin (RBV)<sup>368</sup>.

### **3. HCV treatment outcome and IFN $\alpha$ signaling**

#### **3.1. PegIFN $\alpha$ 2/RBV as HCV treatment**

Combination treatment with pegIFN $\alpha$ /RBV was the standard of care for HCV treatment for the past 25

years. Treatment outcome depends on HCV genotype with 60-80% efficacy for genotype2/3 but only 20-50% for genotype1/4<sup>364</sup>. Such differences may be related to NS5A affinity to phosphorylated STAT1, resulting in variable ISGs expression<sup>364</sup>. Treatment efficacy is also decreasing with the presence of HIV-1 co-infection <sup>369</sup>.

PegIFN $\alpha$  significantly induces 200 to 300 genes in the liver, but nearly 2000 genes in PBMCs <sup>368</sup>. There is an important overlap between PegIFN $\alpha$ 2 regulated genes in liver and PBMC, including Viperin, OAS1, Mda5, USP18, and CCL8. Responsiveness to IFN $\alpha$  therapy has been associated to different factors including pre-activation of the IFN $\alpha$  pathway, IL-28B polymorphism, which will be discussed later in this section. HCV therapy efficacy also depend on the patient's immunological status at the baseline such as pretreatment CD4<sup>+</sup> T cell count<sup>364</sup>, which is much lower for HIV-1-HCV co-infected patients in comparison to HCV mono-infected individuals. Age, gender, body weight, fibrosis, diabetes (obesity), insulin resistance and NK cell responses have also been shown to influence responsiveness to IFN in HCV individuals<sup>364</sup>. Importantly, how IFN treatment leads to clearance of HCV is not entirely clear. PegIFN $\alpha$  may stimulate hepatic DCs or KC to secrete IFN $\lambda$ , which will indirectly sustain the expression of antiviral genes in hepatocytes <sup>352</sup>. PegIFN $\alpha$  injections can also activate STAT1 in non-parenchymal, sinusoidal cells in patients with a pre-activated hepatic IFN system whereas no further increase in phospho-STAT1 signals was found in hepatocytes <sup>368</sup>. The exact pathways triggered by PegIFN $\alpha$  that contribute to HCV control needs to be examined further.

### **3.2. Association between the pre-activation of the IFN pathway and refractiveness to pegIFN $\alpha$ /RBV**

The concept of desensitization of the IFN signal transduction pathways during prolonged exposure of cultured cells to IFN $\alpha$  has been described since the 1980s. It has been emphasized as a mechanism underlying PegIFN $\alpha$  treatment failure in HCV infection. Chen et al. were the first to describe a hepatic gene signature involved in IFN treatment response in HCV-infected individuals <sup>367</sup>. The expression of ISG15, activating transcription factor 5 (ATF5), IFIT1, MX1, USP18, dual specificity phosphatase 1 (DUSP1), involved in the MAPK pathway, HECT and RLD domain containing E3 ubiquitin protein ligase 5 (CEB1) and ribosomal protein S28 (RPS28) were predictable of a sustained virological responses (SVR) in 30 out of 31 patients <sup>367</sup>. Sarasin-Filipowicz and colleagues showed that refractoriness to PegIFN $\alpha$  treatment was associated to a pre-activation of the IFN pathway in the liver as characterized by higher expression of ISGs and phosphorylation and nuclear localization of STAT1 <sup>368</sup>. Higher mRNA level of IFN $\alpha$  receptor was found in HCV-infected responder subjects compared with nonresponders <sup>370</sup>. The capacity of PBMCs IFN pathway profile to predict responsiveness to IFN therapy is conflicting <sup>349,368,371,372</sup> <sup>372</sup>, however, CXCL-10 (also known as IP-10) has become a well-known surrogate marker of ISGs activation and predictor of IFN treatment success<sup>312</sup>.

Activation of negative feedback loops could play a role in the refractoriness to IFN treatment. Higher SOCS3 levels have been observed in HCV infected individuals, particularly in nonresponders to IFN treatment,

than in healthy individuals<sup>364</sup>. In addition, USP18 is upregulated in pretreatment liver biopsies of nonresponders<sup>367,368</sup>. USP18 has been highlighted as an important regulator of the antiviral activity of IFN against HCV infection *in vitro*. Work from Sarasin-Filipowicz and colleagues concluded that SOCS1/3 are responsible for the early termination of IFN $\alpha$  signaling, while USP18 is the key mediator of long-lasting refractoriness to repeated or prolonged stimulation with IFN $\alpha$  in the mouse liver<sup>373</sup>. However, the apparent half-life of USP18 mRNA is 3 to 4 hours. How can then this IFN $\alpha$ -induced negative regulator of IFN $\alpha$  signaling be persistently induced at high levels? One possibility is that very weak STAT1 activity may be sufficient for USP18 induction<sup>373</sup>. An avenue to circumvent lack of responsiveness to IFN $\alpha$  is that IFN $\beta$  and IFN $\lambda$  signaling are not affected by IFN-induced refractoriness *in vivo* and not inhibited by USP18<sup>309</sup>. This is supported by studies showing that pegIFN $\lambda$ 1 is efficient for the treatment of HCV infection in IFN $\alpha$ -treated patients with relapse<sup>374</sup>.

### 3.3 . Impact of the *IFNL* polymorphism on HCV treatment

The impact of *IL28B* (*IFNL3*) polymorphism on HCV treatment response has been extensively studied, including in HIV-1-HCV co-infected patients<sup>355</sup>. Among the identified SNPs, rs12979860 appeared the most relevant, the favorable genotype being associated with about two times higher virologic response. The influence of IL-28B polymorphism on HCV treatment outcome differs according to HCV genotype with a significant association for genotype 1 and 4 but not 2 and 3<sup>355</sup>. Interestingly, IL-28B polymorphisms are also predictive of outcomes for combination therapy DAAs and PegIFN/RBV and even IFN-free treatment. No protective role of IL-28B polymorphism has been observed in HIV-1-disease progression and HIV-1-protection. The functional mechanism associated with *IFNL3* genotype remains elusive. Correlation with expression of *IFNL3* and/or IFN- $\lambda$ 3 or ISGs expression in either PBMCs or in the liver has been controversial and may be related to cell-type specificity<sup>355</sup>. McFarland et al. recently highlighted a mechanism linking *IL28B* SNP with IFN $\lambda$ 3 expression<sup>375</sup>. HCV induced the expression of miR-208b and miR-499a-5p that target *IFNL3* UTR, involved in *IFNL3* mRNA stability and subsequent IFN $\lambda$ 3 expression<sup>375</sup>. SNP rs4803217 with T-to-G substitution in the 3' UTR of *IFNL3* affects A-U rich elements in *IFNL3* where miR-208b and miR-499a-5p binds, resulting in higher mRNA half-life for the favorable *IFNL3*-G genomic variant<sup>375</sup>. Interestingly, favorable versus unfavorable *IFNL3* genotype (rs12979860) also induce differential ISGs expression in normal liver<sup>376</sup>.

Besides, IFN $\lambda$ 4 polymorphism is associated with reduced clearance of the virus and may better predict IFN-based treatment response than *IFNL3* (*IL28B*) associated variants in HCV-HIV-1 co-infected patients<sup>309</sup>. Based on two SNPs, one causing a frameshift in the *IFNL4* gene and one causing a non-synonymous change in the coding region of *IFNL4*, it is possible to stratify patients based on (1) having no IFN $\lambda$ 4; (2) having a fully active IFN $\lambda$ 4-P70; or (3) having a far less active IFN $\lambda$ 4-S70<sup>377</sup>. IFN $\lambda$ 4 appears to have a distinct role to other IFN $\lambda$  family members as its expression is rather associated with the loss of control of chronic HCV infection. The type III IFN receptor is a heterodimer receptor composed of interleukin 28 receptor alpha (*IL28RA*) and interleukin 10

receptor beta (IL10RB). Expression of IFN $\lambda$  receptor 1 expression is very low in control liver biopsy samples, but significantly increased in the setting of chronic viral infections<sup>378</sup>. One IL28RA polymorphism (rs10903035) has been associated with treatment outcome in HCV genotype 1/4 patients but not in HCV genotype 3 patients and this independently of IL28B SNPs<sup>379</sup>. Taken together, all these data support a crucial impact of type I/III IFNs crosstalk on the determination of subsequent antiviral responses.

### 3.4. IFN $\alpha$ signaling in IFN-free regimen

HCV direct-acting antiviral (DAA) therapy agents have been recently licensed and have led to tremendous progresses in the treatment of HCV in terms of both efficacy and toxicity profiles. Nevertheless, high costs remain a major hindrance, notably given that treatment for HIV-1-HCV co-infection often occurs in resource-constrained settings. Linas and colleagues' modeling of the cost-effectiveness of HCV treatment in a hypothetical cohort of 10 millions HIV-1-HCV genotype 1, non-cirrhotic, HCV treatment-naïve individuals treated for HIV-1 according to current guidelines suggested that HCV protease inhibitors should only be used in HIV-1-HCV co-infection after a first trial of PegIFN/RBV treatment<sup>380</sup>. Doctors may treat HIV-1-HCV co-infected cirrhotic patients but defer HCV therapy for those with early-stage fibrosis until generic HCV protease inhibitor available. The consequence of failing an initial course of PegIFN/RBV on efficacy of future treatment remains to be determined<sup>380</sup> but lower efficacy of IFN-free regimens in treatment-experienced HCV mono-infected individuals has been suggested.

Recent work from the group of Anthony Fauci has questioned the relevance of the endogenous IFN system for IFN-free therapy treatment outcome<sup>381</sup>. On-treatment viral clearance was accompanied by rapid downregulation of ISGs in liver and blood as well as the reduction of IP-10 plasma levels, regardless of treatment outcome. Such decrease was paralleled by the reduction of intrahepatic expression of type II and III IFNs and their receptors, in line with a contribution of hepatic type III, rather than type I IFNs to hepatic ISGs expression in HCV-infected patients<sup>326,381-383</sup>. Expression of IFNs mRNAs did not change during treatment and in particular IFN $\lambda$  was not detected in PBMCs. In contrast, expression of type I, II and III IFN receptor genes decreased in PBMCs during treatment. Gene expression of *MX1* in PBMCs did not correlate with baseline viral load. ISGs expression in the blood may be driven by different IFN as compared to in the liver. Importantly, they observed an increase in hepatic *IFNA2* expression in patients achieving SVR, suggesting that reactivation of hepatic type I IFN signaling at the end of treatment may be important for achieving favorable treatment outcome, even with IFN $\alpha$ -free DAA regimens. Mechanisms underlying the protective role of type I IFNs induction in HCV liver disease may include IFN $\alpha$ -induced TRAIL associated with the control of HCV by NK cells<sup>384</sup>. The role of pDCs in the induction of *IFNA2* in the liver remains unclear. These results should be considered carefully given the low numbers of patients included in this study that relapse after treatment.

## 4. pDCs in HCV infection

### 4.1. Reduced numbers of circulating pDCs in chronic HCV infection and trafficking to the liver

Reduced numbers of circulating pDCs have been observed in patients with chronic hepatitis as compared to normal controls<sup>140</sup>. While pDC susceptibility to HCV infection have been suggested by some studies but not others, HCV is unlikely to be responsible for pDC loss. No correlation was found between DC counts and titers of HCV RNA in HCV-infected patients<sup>385</sup>. Of note, DC numbers are restored in patients who had cleared HCV after IFN $\alpha$  therapy<sup>385</sup>. Several lines of evidence support increased pDCs trafficking to the liver in HCV infection. First, increased numbers of pDCs have been described in the livers of HCV patients, including histologically<sup>316,317,340,386-388</sup>. Secondly, the increased levels of CXCR3 and CXCR4 on circulating pDCs from HCV-infected patients compared to healthy controls normalized with therapy<sup>389</sup>, suggesting that HCV itself may be involved in pDCs trafficking. However, loss of circulating DC is associated with the histological grade of liver inflammation<sup>316,385,390</sup>. Lower DC numbers in patients with active hepatitis than in HCV-infected patients with persistently normal levels of serum alanine aminotransferase<sup>385</sup>. pDCs chemotaxis to CXCL12 and CXCL10 correlated with the histological activity index inflammation score<sup>389</sup>. Altogether, these data suggest that pDCs sequestration to the liver may be mainly driven by hepatic inflammation.

### 4.2. pDCs impairment in chronic HCV infection

There is little consensus regarding pDC defects in HCV infection<sup>391</sup>. Increased levels of CD40 and CD83 have been described on blood pDCs in untreated HCV genotype 1-infected patients as compared to healthy controls<sup>389</sup>. Other studies have found that blood pDCs from chronically HCV-infected individuals were functionally competent<sup>390,392-395</sup>. *Ex vivo* exposure of pDCs purified from healthy donors to HCV-infected hepatoma cells produce IFN $\alpha$  but fail to induce phosphorylation of NF- $\kappa$ B resulting in incomplete maturation<sup>396</sup>. TLR9 stimulation of pDCs exposed to the HCV-infected hepatoma cells result in normal phosphorylation of NF- $\kappa$ B, suggesting that cell-associated virus did not actively inhibit TLR-mediated NF- $\kappa$ B phosphorylation<sup>396</sup>. Partial maturation may have consequence for the impaired effector cells reported in chronic HCV infection and associated with low numbers of virus-specific T cells observed in chronic HCV infection<sup>391,397,398</sup>. DCs have been shown to have an impaired capacity to stimulate T cells in chronic HCV infection<sup>399-401</sup>. Specifically, pDCs capacity to stimulate T cells has been shown to be impaired by HCV NS5 as indicated by decreased proliferation of T cells and decreased secretion of IFN $\gamma$ <sup>339</sup>. In addition, altered regulation of HLA class I antigen processing-presenting machinery components in pDC (and cDCs) in chronically HCV-infected patients has been recently reported<sup>390</sup>. However, the absence of overall immunodeficiency in HCV chronic infection and the normal susceptibility to other viral diseases suggest that it is unlikely that HCV impairs DC in their ability to generate a T cell response against itself but not against other viruses<sup>402</sup>. However, DC trapping in the HCV-infected liver may result in their inability to activate T cells in lymph nodes. Functional impairment of DC may be more pronounced

in HIV-1-HCV co-infection<sup>403</sup> and may depend on HCV genotype. Higher expression of co-stimulatory molecules on pDC have been in HIV-1-HCV co-infected patients with HCV genotypes 1 and 4 compared to HIV-1-HCV co-infected patients with HCV genotypes 2 and 3 who exhibit similar pDC activation as healthy donors<sup>404</sup>. In HIV-1-HCV co-infected individuals baseline level of pDC activation correlated with HIV-1 but not HCV viral load<sup>404</sup>.

Reports on defects of pDC capacity to produce IFN $\alpha$  are conflicting. Normal levels of IFN $\alpha$  secretion by pDCs of chronic HCV patients in response to TLR7/9 ligands have been observed by some researchers<sup>392,405</sup> but not others<sup>318,406,407</sup>. HCV was shown to impair pDC production of IFN $\alpha$  in response to synthetic TLR9 agonist but not TLR7 agonists<sup>331,332</sup>. Discrepancies may result from the low numbers of patients examined, differences in viral genotypes, the duration of the infection, varying degrees of disease progression as well as age, sex, alcohol use or co-infection with HIV-1 or hepatitis B virus. Different mechanisms have been proposed to explain this controversial defect in pDC capacity to produce IFN $\alpha$ . Downregulation of IFN $\alpha$ -secreting capacity of pDCs during HCV infection may occur via the induction of TNF $\alpha$  and IL-10 by monocytes and immature DCs, directly or indirectly by induction of apoptosis of pDCs<sup>340,387</sup>. Besides, *in vitro* studies have shown that HCV alters pDC-derived type I IFN production. HCV E2 glycoprotein suppresses IFN signaling through binding to BDCA-2<sup>408</sup>. Cross-linking of CD81 on pDCs with either immobilized HCV-E2 or anti-CD81 mAb, but not soluble HCV-E2 protein, inhibits pDCs response to TLR9 ligand CpG-2216<sup>334</sup>. Stone and colleagues recently showed using the GEN2.2 pDC-like cell line that HCV core protein inhibits pDC-derived type I IFN production through the modulation of IRF7<sup>409</sup>. Importantly, it remains unknown whether pDCs become functionally defective once they have migrated to the infected liver<sup>390</sup>.

### **4.3. pDCs and PegIFN/Rib treatment**

pDCs have been poorly studied in the context of PegIFN/RBV treatment, with conflicting data regarding the association between baseline pDC activation state and therapy outcomes<sup>389,395,404</sup>. High baseline maturation levels on pDCs in HIV-1-HCV co-infected individuals may be associated to subsequent inability to respond to further IFN $\alpha$  stimulation<sup>404</sup>. Interestingly, baseline pDCs chemotaxis to CXCL12 and CXCL10 (IP-10) predicted failure of antiviral response<sup>389</sup>, suggesting a detrimental role of liver-infiltrating pDCs in HCV-mediated immunopathology.

## **5. Increased burden in HCV-HIV-1-coinfection**

### **5.1. Epidemiological data on the reciprocal effect of HCV and HIV-1 on disease progression and mortality**

About 30% of HIV-1-infected individuals in Europe and USA are co-infected by HCV<sup>140</sup>. Accelerated hepatic fibrosis progression, higher rates of liver failure, and death have been reported in HIV-1-HCV co-infection compared to patients with HCV mono-infection<sup>140</sup>. HIV-1 infection negatively impacts every stage of

HCV infection: enhancing HCV transmission, decreasing HCV clearance leading to higher rates of chronic infection and to higher HCV VL<sup>140</sup>. ART has very little impact on HCV VL. Although many studies have found that chronic HIV-1-HCV co-infection is associated with increased morbidity and mortality relative to mono-infection with either virus, a recent meta-analysis of over 30 studies gathering a total of 100,000 patients disproved the existence of an increased risk of mortality in co-infected patients pre-HAART<sup>410</sup>. Before the introduction of effective ART, mortality related to HIV-1 overwhelmed that related to HCV. Nevertheless, the meta-analysis confirmed that in the post-HAART era, co-infection increased by about 35% the risk of overall mortality but not of AIDS-defining conditions<sup>410</sup>. In contrast, the Women's interagency HIV study (WIHS) found an almost two-fold increased AIDS risk among the HIV-1-HCV co-infected women as compared to HIV-1-monoinfected women without a CD4 count <200 cells/ $\mu$ L and for ART-naïve women<sup>411</sup>. HIV-1-HCV co-infected individuals include more intravenous drug users (IDU) than HIV-1 mono-infected individuals due to HCV preferential parenteral transmission. Increased risk of mortality in co-infected individuals as compared to HIV-1 mono-infected individuals was observed after excluding/stratifying patients with IDU. Besides, the impact of HCV infection on immune recovery after ART is conflicting. Slower rates of CD4<sup>+</sup> T cells recovery in co-infected compared to HIV-1 mono-infected individuals have been reported in some studies but others found that HCV does not add to HIV-1-mediated destruction of CD4<sup>+</sup> T cells in co-infected patients<sup>369</sup>.

HCV has been suggested to accelerate the natural course of HIV-1 disease by damaging the innate immune function and aggravating the levels of activation markers and co-receptors on T cells in co-infected patients.

## **5.2. Increased immune activation and inflammation in HIV-1-HCV co-infected individuals**

### **5.2.1. Increased activation, exhaustion and immunosenescence of the T cell compartment**

Conflicting results exist regarding the presence of increased levels of immune activation in HIV-1-HCV co-infected subjects as compared to HIV-1 mono-infected subjects. Some studies have observed significantly higher levels of T cell activation in the peripheral blood in HIV-1-HCV co-infection<sup>412,413</sup> but this observation was not reproduced by others<sup>313,414,415</sup>. ART was also not a confounding factor as Al-Harthi et al. evaluated the difference in CD8<sup>+</sup> T cell activation levels between HIV-1-HCV co-infected women and HIV-1-monoinfected women before and after HAART initiation but found them similar<sup>414</sup>. T cell activation may be related to HCV viral load as Gonzalez and colleagues reported a reduced activation upon therapy-mediated suppression of HCV<sup>412</sup>. Importantly, these studies were all of small size.

PD-1 is a marker for early T cell exhaustion and is characterized by impaired cellular proliferation and enhanced apoptosis but sustained T cell function (cytokine production). T cell immunoglobulin mucin family member 3 (Tim3; also known as HAVCR2) is another exhaustion marker that defines a T cell population distinct from the PD-1 expressing population<sup>416</sup>. Increased levels of exhausted T cells have been reported in HIV-1-HCV

co-infection as compared to HIV-1 mono-infection<sup>415</sup>. The co-expression of Tim-3/PD-1 on CD8<sup>+</sup> T cells but not single expression of either marker has been correlated with the fibrosis score, aspartate aminotransferase [AST]-to-platelet ratio index (APRI), in HIV-1-HCV co-infected individuals under HAART<sup>417</sup>. APRI scores have been validated as a non-invasive test for estimating liver fibrosis in HIV-1-HCV co-infection<sup>369</sup>. HCV-specific T cell immune responses, which are important for the successful control of HCV replication are attenuated in HIV-1-HCV co-infection compared to HCV mono-infection<sup>418,419</sup>. Such attenuation may be related to T cell exhaustion.

CD57 expression on CD8<sup>+</sup> T cells is a marker of proliferative inability and replicative senescence. CD8<sup>+</sup> lymphocytes expressing CD57 expand upon chronic antigenic exposure. Loss of CD28 expression is usually correlated to the gain of CD57 expression. HIV-1-infected individuals exhibit 7.7% more CD28<sup>-</sup>CD8<sup>+</sup> T cells than healthy donors<sup>420</sup>. High levels of CD28<sup>-</sup>CD8<sup>+</sup> have been correlated with HIV-1 disease progression<sup>421</sup>. The release of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF $\alpha$  and IL-6, are potential drivers of viral spread and may underlie the deleterious impact of T cell senescence. Immune activation and CD4<sup>+</sup> T cell function are strongly correlated with immunosenescence<sup>422,423</sup>. Immunosenescent cells express high levels of activation markers such as HLADR and CD38<sup>424</sup>. Antiretroviral therapy generally improves immunosenescence<sup>425,426</sup> but do not restore pre-infection levels<sup>420,427</sup>. In a cohort of young co-infected haemophiliacs, Harcourt and colleagues found that the percentage of CD57<sup>+</sup> HIV-1-specific CD8<sup>+</sup> T cells did not differ between HIV-1-HCV co-infected individuals and HIV-1 mono-infected individuals whereas the presence of the two viruses significantly reduced the expression of CD57 on tetramer-positive cells<sup>428</sup>. In addition, immunosenescent CD8<sup>+</sup> T cells may be implicated in the promotion of hepatic fibrosis. Increased frequency of circulating CD8<sup>+</sup> T cells with senescence marker CD57 (CD8<sup>+</sup>CD45RO<sup>+</sup>CD57<sup>+</sup>) have been observed in cirrhotic patients versus healthy donors<sup>429</sup>. Using a mouse model for toxin-induced nonalcoholic steatohepatitis (NASH), Seth et al found that CD8<sup>+</sup>CD57<sup>+</sup> T cells produce pro-apoptotic cytokines in the liver, and activate hepatic apoptosis as determined by increased Bcl-2 and p53 mRNA expressions<sup>430</sup>. Lack of CD57 expression on T, B or NK cells was associated with reduced apoptosis, fibrosis and NASH progression<sup>430</sup>. Increased CD57 expression in CD8<sup>+</sup> T cells appeared to be due to metabolic oxidative stress<sup>430</sup>. Altogether, these data suggest an increased impairment of T cells in the peripheral blood in HIV-1-HCV co-infection.

### 5.2.2. Microbial translocation

Endothelial cells in the gut act as physical and biological barrier to protect the host from microbial invasion. They secrete multiple small antimicrobial peptides (AMPs) such as defensins, cathelicidins and liver expressed antimicrobial peptide-2 (LEAP-2). The loss of the integrity of the epithelial barrier of the gut observed during HIV-1 infection lead to increased GI permeability resulting in increased microbial translocation into the peripheral circulation and chronic immune activation<sup>160</sup>. Increased translocation of microbial products has also been reported in HCV infection<sup>140</sup>. Microbial translocation is associated with chronic inflammation in both HIV-1-infected and HCV-infected patients<sup>140</sup>. Different mechanisms may underlie the increased microbial

translocation reported in either infections including increased GI inflammation with abundant proinflammatory cytokines production, dysregulation of T cell subsets, decreased microbial clearance, defects in the innate immune responses with potential leakage of bacteria and their products to the systemic circulation and induction of chronic immune activation and inflammation<sup>384</sup>. HIV-1-HCV co-infection is associated with dramatic changes in gene expression in the colonic tissues compared with HIV-1 mono-infection<sup>431</sup>, which may result from HCV replication in the colon tissues or from increased cytokine/chemokine production by remote infection and injury in the liver. Impaired LPS detoxification in the liver could also contribute to high immune activation and inflammation levels observed in HIV-1-HCV co-infected subjects<sup>384</sup>.

## **6. Accelerated hepatic fibrosis in HIV-1-HCV co-infection**

Accelerated hepatic fibrosis progression resulting in higher rates of liver failure and death have been reported in HIV-1-HCV co-infection compared to HCV mono-infection<sup>384</sup>. Progression to cirrhosis is two-to-threefold higher in co-infected than HCV mono-infected patients, and approximately 20-30% of HIV-1-HCV co-infected individuals progress to cirrhosis in less than 20 years<sup>369</sup>. Liver-related mortality, therefore, is the leading cause of death among HIV-1-infected persons in the USA in the ART era.

### **6.1. Evaluation of liver fibrosis in HIV-1-HCV co-infection**

The extent of liver fibrosis is an important factor in prognosis and clinical decision-making in chronic HCV infection. Criteria to estimate fibrosis score have been established by the METAVIR Cooperative Study Group. F0 corresponds to no fibrosis; F1 to portal fibrosis; F2 to periportal fibrosis or rare portal-portal septa; F3 to fibrous septa with architectural distortion but with no obvious cirrhosis (bridging fibrosis) and finally F4 corresponds to definite cirrhosis. Transient elastography (TE) (Fibroscan®), which uses ultrasound readings to measure liver elasticity or stiffness, can accurately stage fibrosis and cirrhosis in HIV-1-HCV co-infected patients<sup>369</sup>. Values 7.0 or less, between 7.1 and 9.4, and between 9.5 and 12.4, and 12.5 or more were considered to correspond with Metavir scores F0-F1, F2, F3, and F4, respectively<sup>432</sup>. Serologic markers have also been used successfully to assess liver fibrosis in HIV-1-HCV co-infected individuals<sup>369</sup>, including the FIB-4 score defined by  $(\text{age}[\text{yr}] \times \text{AST} [\text{U/L}]) / ((\text{platelet count (PLT)} [\text{109/L}]) \times (\text{alanine transaminase (ALT)} [\text{U/L}])^{1/2})$  and the APRI. Of note, FIB-4 < 1.45 defines accurately F0-F1 stage and FIB-4 > 3.25 defines intermediate fibrosis stages but values between 1.45 and 3.25 are poor predictors of intermediate stages. Serum ALT levels are used in monitoring the progression of liver damage. Heavy alcohol intake, increased AST levels, basal liver stiffness are predictors of the severity of fibrosis in HIV-1-HCV co-infected individuals<sup>433,434</sup>. The CD4/CD8 ratio has also been proposed as a non-invasive measurement of liver fibrosis in chronic HCV mono-infected patients<sup>435</sup>. Female sex is associated with a decreased risk to develop fibrosis<sup>234</sup>. Differences in predictors of fibrosis in HCV mono-infected and HCV-HIV-1

co-infected individuals may be due to skewing linked to higher levels of inflammation markers observed in HIV-1-HCV co-infected compared to HCV mono-infected patients.

## 6.2 Mechanisms of hepatic fibrosis

Hepatic fibrosis occurs when there is an imbalance between extracellular matrix (ECM) protein turnover (enhanced synthesis and reduced degradation)<sup>384</sup>. HSCs are the primary source of ECM<sup>384</sup>. They are activated in response to reactive oxygen species (ROS), lipid peroxides, growth factors and inflammatory cytokines produced by hepatocytes or KCs following cell injury<sup>384</sup>. TGF $\beta$ 1 and platelet-derived growth factor (PDGF) are the most influential growth factors in HSC activation and collagen production<sup>384</sup>. Smad7 is involved in this tight restriction of the non-canonical Smad signaling in HSC and regulates the intensity and duration of the TGF- $\beta$  responses<sup>436</sup>. Once activated, HSCs convert into myofibroblast-like cells and will produce inflammatory and pro-fibrogenic mediators as well as secrete ECM including  $\alpha$ -smooth-muscle actin ( $\alpha$ -SMA) and fibrillar collagens (collagens I and III) and tissue inhibitors of metalloproteinases (TIMPs). TIMPs may reduce ECM degradation through suppression of MMP activity by HSCs<sup>384</sup>.

Chronic inflammation is the basis for HCV-mediated liver damage. Particularly, the intrahepatic expression of chemokines ligands and their receptors are important for HCV-mediated liver damage. Elevated intrahepatic levels of RANTES, MIP1 $\beta$ , MIP1 $\alpha$  (ligands for CCR5), IL-6 and TNF $\alpha$  are observed in HCV-infected individuals and have been associated to liver inflammation<sup>384</sup>. CCR5 is involved in the autocrine loop in the fibrogenic response by HSCs<sup>384</sup>. Expression of TNF $\alpha$ , TRAIL and FasL on KCs may contribute to hepatocyte apoptosis and therefore contribute to liver fibrogenesis<sup>384</sup>.

## 6.3 Role of pDCs in liver fibrosis

Hepatic pDCs have a key role in mediating tolerance in the liver<sup>437</sup>. They regulate T cell responses in the liver by different mechanisms including the expression of PD-L1, modulation of Notch ligand expression therefore promoting Th2 responses, IL-10-mediated promotion of Treg differentiation and CD4<sup>+</sup> T cell apoptosis. It was shown that CCR9<sup>+</sup> pDCs abundantly present within the murine liver produce IL-10, and TGF $\beta$  and differentiate naïve T cells to a regulatory phenotype through TLR7 and TLR9 signaling<sup>438</sup>. *In vivo* stimulation with NOD2 ligand leads to upregulation of negative TLR regulator IRF4 in liver but not spleen pDCs, resulting in impaired secretion of pro-inflammatory cytokines and IFN $\alpha$ <sup>437</sup>, showing that exposure to microbial products can inhibit liver pDC function.

Recent works in mice have highlighted a detrimental role of DCs in liver fibrosis. Depleting DCs using a CD11c-DTR transgenic mouse model resulted in delayed fibrosis regression<sup>439</sup>. Using a mice model leptin/thiocetamide model of liver fibrosis, Connolly et al showed that DCs are the primary determinants of the cytokine milieu in the fibrotic liver<sup>440</sup>. Inflammation is a driver for liver fibrosis. Hepatic pDCs produce

inflammatory cytokines in response to TLR7 and TLR9<sup>441,442</sup>. In contrast, DCs can secrete significant levels of MMP in both mice and humans, which will be protective against fibrosis progression<sup>443-445</sup>. Castellaneta and colleagues recently investigated the role of pDCs-derived IFN $\alpha$  in a mouse model of liver ischemia/reperfusion (I/R)<sup>446</sup>. I/R occurs following transplantation, and adversely affects patient and graft outcomes. I/R is characterized by hepatocyte necrosis and is accompanied by inflammation and oxidative damage. In that model, pDCs are activated by self-DNA from necrotic hepatocytes to produce IFN $\alpha$ . IFN $\alpha$  mediates IRF1 upregulation in hepatocytes, promoting Fas-mediated apoptosis of hepatocytes. Selective pDC depletion reduced liver injury after I/R<sup>446</sup>. This data show that pDC-derived IFN $\alpha$  promote liver injury after initial damage of hepatocytes. It is tempting to parallel these findings with HCV-mediated liver fibrosis. Following primary damage of hepatocytes by HCV, pDC-derived IFN $\alpha$  may similarly promote hepatocyte apoptosis. Further research is needed to dissect the role of pDCs in HCV-mediated liver fibrosis.

#### **6.4. Mechanisms of increased hepatic fibrosis in HIV-1-HCV co-infected individuals**

Sociodemographic characteristics of HIV-1-HCV co-infected individuals may represent potential caveats regarding the causes of accelerated hepatic fibrosis. There is a high prevalence of heavy alcohol use in HIV-1-HCV co-infected individuals and alcohol use is related to liver disease progression through the enhancement of mitochondrial damage, local lymphocyte recruitment, gut permeability and translocation of bacterial products such as LPS<sup>384</sup>. In addition, alcohol inhibits the anti-fibrotic action of NK cells<sup>384</sup>. Similarly, drugs such as heroin, cannabis, amphetamine and cocaine cause liver injury and fibrosis progression<sup>384</sup>. We will now detail the various biological mechanisms underlying accelerated hepatic fibrosis progression in HIV-1-HCV co-infected patients<sup>384</sup>. Figure 17 summarizes the mechanisms underlying accelerated progression to fibrosis in HIV-1-HCV co-infection. Those include direct effect of HIV-1, effect of HIV-1 associated dysregulation of immune responses, HIV-1-associated microbial translocation and hepatotoxicity of antiretroviral drugs.

#### **6.5. Hepatic fibrosis as a driver of systemic immune activation and inflammation in HIV-1-HCV co-infection**

Cirrhosis is known to induce global immune dysfunction<sup>447</sup>. Cirrhosis compromises immune cell functions at the systemic level<sup>447</sup>, impairing B cell frequencies and peripheral blood absolute counts, activation and proliferation of circulating T lymphocytes and circulating NK cells function. Besides, T cell lymphopenia is common in cirrhosis and affects both CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>447</sup>. In addition, circulating monocytes are activated *in vivo* in cirrhosis, as determined by enhanced surface expression of HLA-DR, CD80 and CD86, as well as increased production of pro-inflammatory cytokines<sup>447</sup>. Increased serum levels of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, IL-18, IFN $\gamma$ ) or receptors (sTNFR1, IL1sRI, sCD14) are observed in cirrhotic patients<sup>447</sup>, highlighting the increased systemic inflammation in cirrhotic patients. Different mechanisms underlie the

heightened systemic immune activation and inflammation in cirrhosis. First, damaged liver has impaired ability to secrete complement components and secreted PRRs that play a role in the regulation of the immune response<sup>447</sup>. The alteration in intestinal motility, the higher gastric pH and the reduced bile acid concentration in the colon seen in patients with cirrhosis, may participate in the failure in the control of bacterial intestinal growth, favoring leaking from the gut and microbial translocation<sup>447</sup>. Portal hypertension, a situation typical of advanced phases of hepatopathy, may also increase bacterial translocation. Increased bacterial translocation increases PAMPs availability while hepatocellular damage increases DAMPs presence, both causing the activation of immune cells in the GALT and in the peripheral blood<sup>447</sup>. Immune cells already activated in the GALT and mesenteric LN may enter the peripheral blood and spread the inflammatory response systemically<sup>447</sup>. The inflammatory response secondary to the higher levels of circulating bacteria or PAMPs is among the leading causes of multi-organ failure and death in cirrhotic patients<sup>447</sup>.

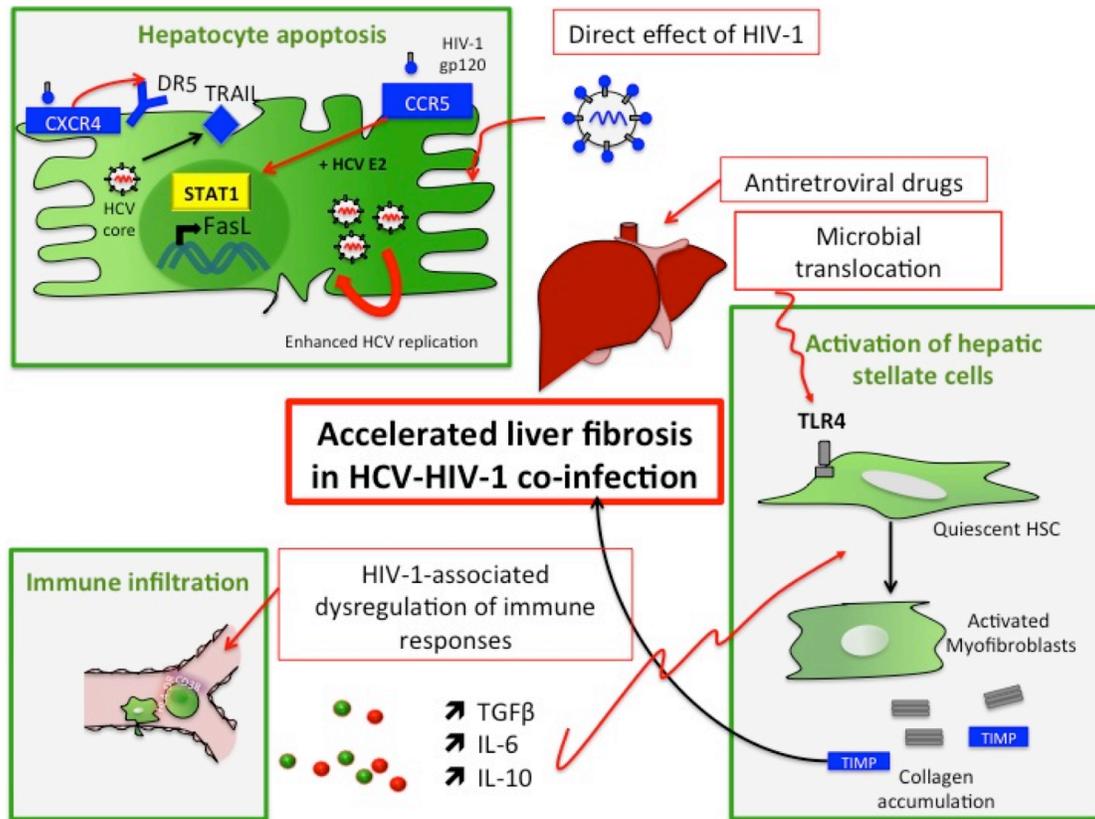
Global immune dysfunction might not occur in less advanced fibrosis stage. However, analysis of the strategies for management of anti-retroviral therapy (SMART) study, designed to assess risk of death in patients undergoing CD4<sup>+</sup> guided treatment interruptions, found that an excess risk of death in HIV-1-HCV co-infected individuals compared to HIV-1 mono-infected individuals was confined to persons with pre-existing subclinical signs of liver impairment as determined by levels of hyaluronic acid<sup>448</sup>. Subsequent analysis found that HIV-1-HCV co-infected persons with impaired liver function are in an enhanced proinflammatory state as determined by IL-6 and sCD14 plasma levels<sup>449</sup>. Altogether, these data suggest that impaired liver function, at least for advanced stages of fibrosis, perturbs systemic inflammation and immune activation. HIV-1-mediated acceleration of liver fibrosis progression may therefore participate in a vicious circle where advanced fibrosis further amplifies systemic inflammation and immune activation.

## **7. Sex differences in HIV-1-HCV co-infection**

It is well established that female sex is a predictor of spontaneous clearance of acute HCV infection<sup>234</sup>. The mechanisms behind the association of female sex and HCV spontaneous clearance might be linked to sex-based differences in immunity. For instance, it has recently been shown that the effect of the *IL28B* genotype on clearance was greater among women than among men<sup>450</sup>. This sex-based differences in immunity might also be partially responsible for the better control of HIV-1 replication in acute infection leading to the lower viral setpoints observed in HIV-1-infected women than in HIV-1-infected men. It has been postulated that HCV clearance in women might be facilitated by estrogens<sup>234</sup>. However, HIV-1-HCV-co-infection has been associated to HCV persistence<sup>384</sup>. It is unknown whether HCV clearance rate are higher in HIV-1-HCV-coinfected women as compared to HIV-1-HCV-coinfected men.

In the HIV-1-HCV co-infected population, it has been reported that women have higher mortality rates than men despite similar viral suppression and CD4<sup>+</sup> T cell count, even when controlling for IDU history, race,

and baseline CD4<sup>+</sup> T cell count<sup>451</sup>. The higher mortality rates in HIV-1-HCV co-infected women were not due to sex differences in virological and immunological responses to ART<sup>451</sup>. Sex differences in mortality could be due to more rapid HCV-related liver disease in women as suggested by Rodriguez-Torres and colleagues. They showed that HIV-1-HCV-co-infected women had a median survival time to cirrhosis of 16.0 years less than HCV-mono-infected women while there was no difference in the risk for cirrhosis between HCV-mono-infected and co-infected men<sup>452</sup>. However, those results might have been biased by an increased use of alcohol by HCV-mono-infected men<sup>452</sup>. In contrast, Collazos and colleagues have demonstrated that HIV-1-HCV-co-infected women have more favorable HCV virological and clinical profiles than men and, particularly, lower degrees of fibrosis<sup>453</sup>. In HCV mono-infection, it is well recognized that men have higher rate of disease progression compared to women<sup>454</sup>. Differences in treatment discontinuation between HIV-1-HCV co-infected women and HIV-1-HCV co-infected men might also potentially explain the observed sex differences in mortality rates. In HIV-1-HCV co-infection, adverse events during HCV therapy account for 12-39% of treatment discontinuations and occur more frequently in HIV-1-infected women during HCV therapy than HIV-1-infected men<sup>455</sup>. Generally, women including HCV-infected women and HIV-1-infected women, experience more adverse events in response to drug therapies than men<sup>234</sup>. For instance, HCV-infected women are more likely to experience anemia<sup>456</sup> and develop depression<sup>457,458</sup> than HCV-infected men upon IFN/RBV treatment, which was until very recently the standard for HCV therapy. Depression is more frequent in HIV-1-infected women and women with viral hepatitis than men<sup>459-462</sup> and is an important reason for therapy interruption in women<sup>451</sup>. The combination of HCV therapy and some types of ART regimen might specifically enhance the adverse event rates in HIV-1-HCV co-infected women compared to HIV-1-HCV co-infected men<sup>455</sup>. Altogether, these data suggest that sex differences in disease progression in HIV-1-HCV co-infection require further investigation.



**Figure 17: Mechanisms underlying accelerated progression to fibrosis in HIV-1-HCV co-infection**

HIV-1 may directly enhance liver fibrosis via cytopathic effect on hepatocytes, which express the HIV-1 coreceptors CXCR4 and CCR5, or via the enhancement of HCV replication. HCV-E2 protein and HIV-1 gp120 may collaboratively induce FasL-mediated hepatocyte apoptosis via STAT1 pathway. In HCV-HIV-1 co-infection, increased hepatocyte apoptosis may also occur through the simultaneous HIV-1-mediated increase of DR4/5 via CXCR4 ligation on hepatocytes, together with HCV core-mediated induction of hepatocyte-derived TRAIL. Antiretroviral drugs, particularly with strong mitochondrial toxicity, have been associated with increased liver fibrosis progression in HIV-1-HCV co-infected patients. Microbial translocation can result in the activation HSCs via TLR4, therefore leading to liver fibrosis. HIV-1-associated dysregulation of immune responses may also be involved in the acceleration of liver fibrosis via enhanced production of pro-fibrotic cytokines such as TGF- $\beta$ 1, IL-10 or IL-6 levels. Similarly, alterations of chemokines networks may also enhance liver fibrosis by enhancing immune infiltration of activated cells.

*Adapted from [384]*

Type I IFNs signaling with important modulation by type III IFNs has therefore clinical importance in HIV-1-HCV co-infection. The mechanisms underlying the role of pDCs-derived type I IFNs in HIV-1-HCV co-infection, notably as drivers of increased immune activation and inflammation are insufficiently understood and will be explored in this thesis.



## **Hypothesis and specific aims**



## Hypothesis and specific aims

Abnormal pDC IFN $\alpha$  production plays an important role in HIV-1 immunopathogenesis. Given that IFN $\alpha$  levels only partially normalize upon treatment, its deleterious effect likely persists even under suppressive ART. Furthermore, IFN $\alpha$ -driven inflammation and immune activation may be involved in the development of non-AIDS related events including cardiovascular and liver disease in the post-ART era. Several mechanisms underlie the detrimental role of IFN $\alpha$  in HIV-1 immunopathogenesis, in line with the multifaceted actions of IFN $\alpha$  on the immune system. As discussed above, the molecular mechanisms associated to a given immunomodulatory effect are incompletely understood. Moving forward, a better understanding of the pathways involved is required. Centered on this challenging question, my thesis articulates around two axes as detailed below.

### Specific aim 1

pDCs from healthy females produce more IFN $\alpha$  upon TLR7 stimulation compared to pDCs from healthy males. We hypothesized that naturally-occurring sex difference in IFN $\alpha$  production may provide a physiological model to investigate the mechanisms capable to control IFN $\alpha$  production without inducing unwanted pathogenic effects. Furthermore, sex differences have been reported throughout the natural course of HIV-1 infection and seem to be mainly organized around differences in IFN $\alpha$  production and immune activation. The mechanisms underlying the difference in IFN $\alpha$  production between men and women have been incompletely dissected but there is consensus in the involvement of the ER $\alpha$  signaling. Informative parallels with systemic lupus erythematosus, an autoimmune disease characterized by a strong sex bias and pathogenic IFN $\alpha$ , can be drawn. IRF5 polymorphisms, associated notably with increased expression, have been associated to SLE. Thus, we studied the contribution of IRF5, together with the master regulator of type I IFNs IRF7, to sex difference in pDC IFN $\alpha$  response to TLR7 stimulation in healthy donors. Our results are presented in study 1.

### Specific aim 2

HCV co-infection is common in HIV-1-infected individuals and is the driver of one of the most prevalent mortality cause among HIV-1-infected individuals (liver disease). Several factors make the study of HCV-HIV-1 co-infection relevant to assess the impact of IFN $\alpha$  signaling modulation in a physiological, or, here, rather pathophysiological context. First, IFN $\alpha$  has been used as therapeutic agent against HCV for many years. Importantly, IFN $\alpha$ -based treatment failure is associated with pre-activation of the IFN pathway, highlighting a major hindrance to any therapeutic strategy aiming at the modulation of type I IFN responses. The pre-treatment state of activation of the type I IFN pathway modulate treatment outcomes. Then, HCV itself may not lead to high immune activation

levels, although indirect effects mediated by cirrhosis-driven inflammation are recognized. Nevertheless, increased levels of immune activation and/or inflammation have been reported in HIV-1-HCV co-infected individuals as compared to HIV-1 mono-infected individuals, with inconsistency though. Both viruses can activate pDC IFN $\alpha$  response via TLR7. HCV may therefore act as a factor mediating increased IFN $\alpha$  levels, as reported by one study. In contrast, both HIV-1 and HCV hijacks various components of IFN $\alpha$  signaling in an effort to subvert antiviral responses. How these alterations affect the global IFN $\alpha$  response remains to be determined. We hypothesized that the integrated analysis of defects of type I IFN signaling in pDCs both at the transcriptomic and protein levels together with impairments associated to immune activation and inflammation may lead to the identifications of potential targets in IFN $\alpha$  signaling that are associated with immune activation/inflammation. Finally, the emergence of IFN-free treatment against HCV allows the evaluation of the strict impact of HCV viral clearance on the restoration of IFN $\alpha$  pathway and immune responses. Our results are reported in study 2

## Results



## Study n°1

### **Sex differences in plasmacytoid dendritic cell levels of IRF5 drive higher IFN $\alpha$ production in women**

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Sex differences in TLR7-mediated pDC IFN $\alpha$  production were initially reported by Berghofer and colleagues<sup>289</sup>. Our group was the first to highlight a link with stronger immune activation<sup>187</sup>, therefore providing a mechanism by which the same level of viral replication might result in faster HIV-1 disease progression in women compared to men<sup>236</sup>. Hence, our group participated in the groundbreaking work that evidenced the pathogenic role of IFN $\alpha$  in chronic HIV-1 infection, which has been initiated by others a few years earlier<sup>147</sup>. The present study is the follow-up of this pioneer study. Our goal was to understand what molecular mechanisms were driving increased IFN $\alpha$  production in females compared to males. Before our study, the group of Jean-Charles Guéry provided fundamental clues by demonstrating a role of ER $\alpha$  signaling<sup>259</sup>. Importantly, most of their work was based on mice model. Here, we uncover the role of IRF5 in sex differences in pDC IFN $\alpha$  production, based principally on primary human pDCs.

Addressing functional mechanisms in human pDCs is difficult due to their scarcity. For this project, we have invested time and efforts implementing several novel techniques to address specific questions. First, we redirected the TissueFAXS slide scanner from its primary use (tissue scanning) in order to provide quantitative measures of IRF5 translocation in biological settings. Indeed, as bulk PBMCs were used, there was no requirement for stressful or activating enrichment procedures. In addition, pDCs could be stimulated in an environment allowing for potentially relevant cell-cell interactions with other PBMCs subsets. Then, we utilized a novel vector-free technique, referred as “squeezing” to mechanistically address IRF5 contribution to IFN $\alpha$  transcription. pDCs IFN $\alpha$  responses to synthetic TLR7 ligand are time-sensitive<sup>463</sup>. Accurate measures are usually obtained when the stimulation is started within the 5-6 hours following venipuncture. Days long cultivation of pDCs required for siRNAs-mediated silencing experiments may not be compatible with the time-sensitivity of pDCs TLR7-mediated IFN $\alpha$  responses. We therefore decided to “squeeze” recombinant IRF5 protein into primary pDCs. Delivery of IRF5 but not TUBA1A, not involved in the pDCs TLR7/IFN $\alpha$ , lead to significant increase in the percentage of IFN $\alpha$ -producing pDCs. Importantly, while the use of primary pDCs may prevent the extent to what can be mechanistically assessed, it is the most physiologically relevant model. Indeed, pDC-like cell lines exhibit major differences compared to primary pDCs in regard of TLR7-induced IFN $\alpha$  production. Finally, we evaluated the impact of ER $\alpha$  on IRF5 regulation. We show that pDCs from mice with a conditional knockout of ER $\alpha$  in DC lineage have a decreased IRF5 expression as well as impaired IFN $\alpha$  responses to TLR7 stimulation, as compared to pDCs from WT mice. We use the novel FlowRNA method to investigate correlation between ER $\alpha$  and IRF5 expression in humans.

In conclusion, this study highlights for the first time a mechanism by which increased IRF5 levels in pDCs from females, controlled by ER $\alpha$  signaling, drives increased IFN $\alpha$  upon TLR7 stimulation.

# Sex differences in plasmacytoid dendritic cell levels of IRF5 drive higher IFN $\alpha$ production in women

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**RUNNING TITLE: Sex difference in pDC IRF5 expression**

## ABSTRACT

Increased interferon alpha (IFN $\alpha$ ) production contributes to the pathogenesis of infectious and autoimmune diseases. Plasmacytoid dendritic cells (pDCs) produce more IFN $\alpha$  upon toll-like receptor 7 (TLR7) stimulation in females than in males, yet the mechanisms underlying this difference remain unclear. Here, we show that basal levels of interferon regulatory factor 5 (IRF5) in pDCs were significantly higher in females compared to males and correlated to the percentage of IFN $\alpha$ -secreting pDCs. Delivery of recombinant IRF5 protein into human primary pDCs increased TLR7-mediated IFN $\alpha$  secretion. In mice, genetic ablation of the estrogen receptor 1 (*Esr1*) gene in the hematopoietic compartment or DC lineage reduced *IRF5* mRNA expression in pDCs and IFN $\alpha$  production. *IRF5* mRNA levels furthermore correlated with *Esr1* mRNA levels in human pDCs, consistent with *IRF5* regulation at the transcriptional level by *Esr1*. Taken together, these data demonstrate a critical mechanism by which sex differences in basal pDC IRF5 expression lead to higher IFN $\alpha$  production upon TLR7 stimulation in females, and provide novel targets for the modulation of immune responses and inflammation.

## INTRODUCTION

Significant differences in immune responses between females and males, including responsiveness to vaccination (Klein et al, 2010), have been long noted (Green et al, 1994; Grossman, 1985; Mitchell, 1999; Olsen & Kovacs, 1996) but often remain overlooked in immunological studies and particularly in studies of human viral infections (Klein, 2000), as most studies have been carried out in rodents (Boissier et al, 2003; Cao et al, 2008; Hannah et al, 2008; Weinstein et al, 1984). As a general rule, females exhibit more robust cell-mediated and humoral immune responses to antigenic challenges compared to males (Hewagama et al, 2009; Klein et al, 2010; van Lunzen & Altfeld, 2014; Villacres et al, 2004). Furthermore, females are also often more prone to immune-mediated pathology and autoimmunity (Voskuhl, 2011). The heightened inflammatory immune responses observed in females have been suggested to contribute to sex differences in the clinical manifestations, immune responses and outcome of viral diseases, including influenza A virus (Klein, 2012), hantavirus (Klein et al, 2011), hepatitis C virus (HCV) (Grebely et al, 2014; van den Berg et al, 2011) and human immunodeficiency virus (HIV-1) (Addo & Altfeld, 2014; Farzadegan et al, 1998) .

The pathways underlying these sex differences in the manifestations of viral and autoimmune diseases are not well understood, but increasing data suggest a critical role of the toll-like receptor 7 (TLR7) pathway and resulting type I interferon (IFN) production in the outcome of these diseases (Bao & Liu, 2013; Di Domizio et al, 2009; Ganguly et al, 2009; Teijaro et al, 2013; Wilson et al, 2013). Our group and others have previously shown that plasmacytoid dendritic cells (pDCs) derived from females produced significantly more IFN $\alpha$  in response to TLR7 ligands than pDCs derived from males, resulting in stronger immune activation (Berghofer et al, 2006; Meier et al, 2009), and that sex hormones can regulate the IFN $\alpha$  response to TLR7 stimulation (Seillet et al, 2012; Seillet et al, 2013). However, the mechanisms underlying this sex difference in TLR7-induced IFN $\alpha$  production by pDCs remain unknown.

IFN $\alpha$  induction is regulated primarily at the transcriptional level by the interferon regulatory factors (IRF) family (Honda et al, 2006; Ozato et al, 2007; Taniguchi & Takaoka, 2002). In response to stimulation, these transcription factors are phosphorylated on serine residues, a modification that stimulates protein dimerization,

nuclear translocation, and interaction with transcriptional co-activators (Barnes et al, 2001; Honda et al, 2005). pDCs express high constitutive levels of IRF5 as well as IRF7 (Coccia et al, 2004; Izaguirre et al, 2003; Schoenemeyer et al, 2005) and TLR7 activation of pDCs leads to the activation and phosphorylation of both IRF5 and IRF7 (Barnes et al, 2001; Kumar et al, 2000; Marie et al, 2000; Sato et al, 2000). IRF7 is widely recognized as the 'master regulator' of type I IFN (Honda et al, 2005), while IRF5 has been shown to be a central mediator of TLR7 signaling (Schoenemeyer et al, 2005; Takaoka et al, 2005). In addition, IRF5 polymorphisms have been associated with multiple autoimmune diseases, and in particular systemic lupus erythematosus and rheumatoid arthritis (Dieguez-Gonzalez et al, 2008; Graham et al, 2006; Nordang et al, 2012; Sigurdsson et al, 2007), two autoimmune diseases characterized by significant sex differences in prevalence. Autoimmune-risk haplotypes exhibit higher IRF5 levels (Diaz-Lagares et al, 2011; Feng et al, 2010) and are associated with increased levels of IFN $\alpha$  (Hedl & Abraham, 2012; Niewold et al, 2008; Rullo et al, 2010), suggesting that expression of IRF5 contributes to the development of autoimmune diseases (Clark et al, 2013).

In this study, we investigated the role of IRF5 and IRF7 in the difference in IFN $\alpha$  production observed between females and males. Our results demonstrate that IRF5 levels are regulated by the estrogen receptor  $\alpha$  (ER $\alpha$ ) in mice, and that sex difference in IRF5 expression in human pDCs can lead to higher IFN $\alpha$  production in females compared to males following TLR7 stimulation, providing potential novel targets for the modulation of inflammation and immune responses in both chronic viral and autoimmune diseases.

## RESULTS

### Sex differences in the IFN $\alpha$ /TLR7 pathway in pDCs

Our group and others have previously reported that pDCs derived from females produce markedly more IFN $\alpha$  in response to TLR7 ligands than pDCs derived from males (Berghofer et al, 2006; Meier et al, 2009; Seillet et al, 2012). These results were confirmed here by measuring the frequency of IFN $\alpha$ -producing pDCs in a first group of 31 healthy individuals (17 females, 14 males) (Table EVI). A significantly higher percentage of IFN $\alpha$ -producing pDCs after 20 hours of stimulation with the synthetic TLR7/8 ligand CL097 was observed in females than in males (Figure 1A,  $p=0.04$ , two-tailed Mann-Whitney test). Neither age nor ethnicity influenced IFN $\alpha$  production by pDCs ( $p=0.1$ ,  $r=0.3$ , Spearman rank-based correlation;  $p=1.0$ , Fisher Exact test). The mean frequency of IFN $\alpha$ -producing pDCs was 50.15% in females and 39.53% in males, in line with previous reports (Meier et al, 2009). In contrast, no sex difference was noticed in the percentage of TNF $\alpha$ -producing pDCs (Figure 1A,  $p=0.54$ , two-tailed Mann-Whitney test).

Little is known about the mechanisms underlying the observed sex difference in IFN $\alpha$  production. IRF7 and IRF5 are two crucial transcription factors activated upon TLR7 stimulation that modulate IFN $\alpha$  production (Schoenemeyer et al, 2005). *Ex vivo* levels of IRF5 and IRF7 in pDCs were measured subsequently by flow cytometry in a second group of healthy donors (Table EVI). No sex difference was observed in the *ex vivo* levels of IRF7 in pDCs (Figure EV1A,  $p=0.64$ , two-tailed Mann-Whitney test). In contrast, pDCs derived from females contained 1.6 times more IRF5 than pDCs derived from age- and ethnicity-matched males, as measured by the MFI level of *ex vivo* IRF5 expression (Figure 1B, females  $n=29$ , males  $n=19$ ;  $p=0.02$  two-tailed Wilcoxon Rank test), and also observed by western blot in pDCs pooled from five female donors as compared to five male donors (Figure EV1B). IRF5 expression among pDCs appeared heterogeneous with some pDCs expressing no or very low levels of IRF5 as determined by the use of an isotype control (Figure 1C). Of note, no difference in IRF5 protein levels was noticed between pre-menopausal females under hormonal birth control ( $n=8$ ) and those without hormonal birth control ( $n=11$ ). *Ex vivo* expression levels of IRF5 protein were also examined in CD3<sup>+</sup> T cells, in which the MFI of IRF5 was significantly lower than in pDCs ( $p<0.0001$ , Wilcoxon matched-pairs signed

rank test), and did not significantly differ between females and males (Figure 1B,  $p=0.26$ , two-tailed Mann-Whitney test), suggesting that the sex difference in *ex vivo* levels of IRF5 is cell-type specific. These results highlight a potential role of IRF5 levels but not IRF7 levels in the observed sex differences in IFN $\alpha$  production.

### **Sex difference in basal IRF5 protein levels can influence pDC responses to TLR7 stimulation**

To gain a better understanding of the biological consequences of the sex difference in basal protein levels of IRF5 in pDCs, IRF5 subcellular localization after TLR7 stimulation was measured using the TissueFAXS slide scanning system. PBMCs were stimulated for 2 hours with CL097 before staining for IRF5. pDCs were identified among all of the plated cells as HLA-DR<sup>+</sup>CD123<sup>+</sup> (Figure 1D), and the levels of IRF5 in both the cytoplasm and nucleus from all pDCs on the slide was subsequently quantified. The ratio of nuclear IRF5 to cytoplasmic IRF5 was calculated to determine the changes in IRF5 translocation to the nucleus. Higher ratios of IRF5 in the nucleus after stimulation were observed in females than in males (Figure 1E;  $p=0.11$ ; two-tailed Mann-Whitney test). The total amount of IRF5 among pDCs did not vary between baseline and after 2 hours of stimulation with CL097 ( $p=0.19$ , paired Mann-Whitney test). This suggests that the sex difference in IRF5 levels observed prior to stimulation might translate to higher nuclear IRF5 content following TLR7 stimulation.

The consequences of the sex difference in basal levels of IRF5 in pDCs on IFN $\alpha$  production by pDCs were subsequently examined by measuring *ex vivo* IRF5 levels and the percentage of IFN $\alpha$ -secreting pDC by flow cytometry. *Ex vivo* IRF5 levels prior to stimulation positively correlated with the percentage of IFN $\alpha$ -secreting pDCs after 2 hours of stimulation with CL097 (Figure 2,  $r=0.8$ ,  $p=0.02$ , Spearman rank-based correlation), demonstrating a link between the basal quantity of IRF5 and the production of IFN $\alpha$ .

### **Delivery of exogenous IRF5 protein increases IFN $\alpha$ secretion in response to TLR7 stimulation in pDCs**

We further examined the direct impact of IRF5 protein levels on IFN $\alpha$  production. Given that pDCs left in culture for more than 6 hours have significantly reduced capabilities to produce IFN $\alpha$  in response to TLR7 stimulation (Meier et al, 2008), techniques involving long incubation period such as siRNA or transfection of

vectors containing IRF5 are not ideal. Here, we adapted a technique recently described by Sharei and colleagues (Sharei et al, 2013b) for the direct delivery of IRF5 recombinant protein into human primary pDCs. A microfluidic device injects cells in narrow lanes inducing cell constriction, creating transient holes in plasma membranes and enabling passive entry of molecules. Different microfluidic devices (described in Table EVII) were tested as the size of the constriction and the number of constrictions were previously shown to influence delivery efficiency (Sharei et al, 2013b). The best delivery efficiency without significant loss of cell viability was obtained using the 10-4x5iS chip where 10 is the length of constriction in  $\mu\text{m}$ , 4 is the width of the constriction in  $\mu\text{m}$  and 5 is the number of times the constriction is repeated through each channel (Figures EV2B and EV2C). pDCs subjected to this optimized delivery method exhibited decreased IFN $\alpha$  secretion in response to TLR7 ligand compared to untreated pDCs, while no unspecific IFN $\alpha$  production was induced in the absence of TLR7 ligand. The 10-4x5iS device enabled efficient delivery of IRF5 protein into live primary pDCs (Figure 3A and Figure EV2D), allowing to assess the impact of IRF5 protein delivery into primary pDCs on IFN $\alpha$  production. Figure 3B shows representative flow plots of IFN $\alpha$ -secreting pDCs with or without IRF5 protein delivery. IRF5 delivery resulted in a significant increase (3-fold) in IFN $\alpha$  production by pDCs (Figure 3C,  $n=7$ ,  $p=0.02$ , Wilcoxon signed rank test). In contrast, delivery of TUBA1A protein, a protein irrelevant for the pDC TLR7/IFN $\alpha$  pathway, did not lead to changes in IFN $\alpha$  production (Figure 3C).

Apart from its role in IFN $\alpha$  production, IRF5 has been described to also promote transcription of pro-inflammatory cytokines such as IL-6 and TNF $\alpha$  upon TLR stimulation (Takaoka et al, 2005). Supernatants were therefore collected after 2 hours of CL097 stimulation from control pDCs and pDCs in which IRF5 protein was overexpressed, and cytokines were measured using the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit. Consistent with previous work (Birmachu et al, 2007), 2 hours of CL097 stimulation did not induce the production of IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-15, IL-17, IFN $\gamma$ , MIG, and eotaxin, whereas TNF $\alpha$ , IL-8, MIP-1 $\alpha$  and MIP-1 $\beta$  protein secretion was induced. pDCs in which IRF5 protein was overexpressed exhibited increased TNF $\alpha$ , IL-8, MIP-1 $\alpha$  and MIP-1 $\beta$  production (Figure 3D). Overall, we showed increased production of IFN $\alpha$  and other inflammatory cytokines following delivery of exogenous IRF5 protein into primary human pDCs using a

novel method of protein delivery, confirming the role of IRF5 in mediating IFN $\alpha$  production.

### **Sex difference in IRF5 levels in pDCs is associated to oestrogen signaling**

Previous reports have shown that ER $\alpha$  signaling can modulate IFN $\alpha$  production by pDCs both in mice (Seillet et al, 2012) and in humans (Laffont et al, 2014). Studies have also shown higher IRF5 mRNA levels in splenic B cells derived from female than in age-matched male mice and from wild-type (WT) mice as compared to ER $\alpha$  knock-out mice (Shen et al, 2010). The analysis of the IRF5 promoter/enhancer region using the ENCODE database revealed an ER $\alpha$  binding site 17kb upstream of the transcription start site for human IRF5. We therefore hypothesized that the observed difference in IRF5 levels of pDCs between the sexes might be related to a transcriptional regulation of IRF5 through ER $\alpha$  signaling. ER $\alpha$ -dependent regulation of IRF5 was investigated in female mice with tissue-specific deletion of ER $\alpha$  (Lelu et al, 2011; Seillet et al, 2012). Before assessing levels of *IRF5* mRNA in pDCs, and in agreement with previous works (Shen et al, 2010), we observed a lower expression of IRF5 protein in splenic B cells from Tie2-ER $\alpha^{\text{KO}}$  mice, lacking ER $\alpha$  in the hematopoietic compartment, as compared to ER $\alpha^{\text{flox/flox}}$  WT mice (Figure EV3A,  $p=0.04$ , unpaired t test) (Shen et al, 2010). We further demonstrated for the first time that bone marrow pDCs derived from unmanipulated Tie2-ER $\alpha^{\text{KO}}$  female mice and CD11c-ER $\alpha^{\text{KO}}$  female mice which specifically lack ER $\alpha$  in dendritic cells, produce less IFN $\alpha$  in response to TLR7 stimulation than pDCs from ER $\alpha^{\text{flox/flox}}$  WT littermate controls (Figure EV3C,  $p=0.02$  and  $p=0.006$  respectively, unpaired t test). This impaired capacity of bone marrow derived pDCs from Tie2-ER $\alpha^{\text{KO}}$  mice and from CD11c-ER $\alpha^{\text{KO}}$  mice to produce IFN $\alpha$  in response to TLR7 engagement was associated with significantly less *IRF5* mRNA expression than in pDCs from WT mice (Figure 4B,  $p=0.003$  and  $p=0.007$ , respectively, unpaired t test). These results demonstrate that IRF5 expression correlates with the level of TLR7-mediated IFN $\alpha$  production in pDCs and is, at least partially, regulated by ER $\alpha$  signaling.

To address the question of ER $\alpha$ -dependent regulation of the *IRF5* gene in humans, *Esr1* (ER $\alpha$  gene) and *IRF5* mRNA transcripts were quantified in the same pDC derived from humans using a novel flow cytometry-based *in situ* hybridization assay, the QuantiGene FlowRNA Assay. This assay based on the use of specific

oligonucleotide probes coupled with branched DNA signal amplification offers the advantage over standard qRT-PCR techniques to control for the potential heterogeneity in the expression among a defined cell type. Figure 4C and D show primary flow plots and histograms of the simultaneous detection of *IRF5* mRNA and *Esr1* mRNA. A significant correlation between the *Esr1* and *IRF5* transcripts was observed in pDCs derived from females but not in pDCs derived from males (Figure 4E; females:  $n=10$ ,  $r=0.81$ ,  $p=0.004$ ; males:  $n=7$ ,  $r=0.45$ ,  $p=0.31$  Spearman correlation). Potential non-specific interactions between the type1-*Esr1* and type4-*IRF5* probes were controlled for by simultaneously testing two different probes (type4-*TLR7* and type1-*TLR9*) coupled to the same fluorophores as used for *Esr1* and *IRF5* probes. Overall, these data demonstrate that estrogen-dependent regulation of *IRF5* transcription via ER $\alpha$  can result in sex differences in *IRF5* levels of pDCs, and downstream IFN $\alpha$  production.

## DISCUSSION

Important differences exist between males and females in the outcome of infectious diseases and occurrence of autoimmune diseases for which the pDCs IFN $\alpha$  response has been implicated in the pathology of the disease. Sex differences in IFN $\alpha$  production by pDCs upon TLR7 stimulation have been previously described by our group and others (Berghofer et al, 2006; Meier et al, 2009; Seillet et al, 2012). Here, we investigated the role of two key regulators of IFN $\alpha$  production, IRF7 and IRF5, in mediating these sex differences in IFN $\alpha$  production. While no sex-based difference in IRF7 expression was observed, significantly higher protein levels of IRF5 were detected in pDCs derived from females than in pDCs derived from males. Basal protein levels of IRF5 were directly associated with the level of IFN $\alpha$ -secretion by TLR7-stimulated pDCs and experimental increase in IRF5 protein levels resulted in increased IFN $\alpha$ -secretion by human pDCs. Bone marrow derived pDCs from mice with conditional ER $\alpha$  knock-out exhibited impaired capacity to produce IFN $\alpha$  in response to TLR7 engagement and significantly less *IRF5* mRNA expression than pDCs from WT mice. Altogether, these results suggest sex hormone-mediated regulation of IRF5 levels in pDCs as an underlying mechanism for the higher percentage of IFN $\alpha$ -secreting pDCs in females as compared to males following TLR7 stimulation.

Previous studies have reported conflicting results regarding the role of IRF5 in IFN $\alpha$  transcription (Lin et al, 2005; Schoenemeyer et al, 2005; Takaoka et al, 2005; Yanai et al, 2007). These studies differed in the cell type and stimulants used, suggesting that the impact of IRF5 on type I IFNs transcription may be cell-type- and stimulus-specific. In this study, the basal levels of IRF5 were found to be significantly higher in pDCs derived from females than in those derived from males. IRF5 was furthermore observed to be functional upon TLR7 stimulation, with IRF5 levels prior to stimulation being correlated with the percentage of IFN $\alpha$ -secreting pDCs after two hours of TLR7 stimulation. However, it should be noted that a subset of IFN $\alpha$ -secreting pDCs with undetectable IRF5 levels was also present. This observation suggests that IRF5 alone does not account for all the IFN $\alpha$  produced by pDCs and may reflect the well-recognized role of IRF7 in IFN $\alpha$  production. Altogether, this data demonstrates that sex difference in basal levels of IRF5 can drive sex differences in IFN $\alpha$  production upon TLR7 stimulation. Although sex-based differences were observed in pDCs, no sex difference in IRF5 expression

was observed in T cells, confirming a cell-specific role of IRF5 in IFN $\alpha$  transcription. A potential explanation for the cell-specific differences in IRF5 might be the presence of multiple alternatively spliced isoforms of IRF5. These different IRF5 isoforms have been shown to have distinct cell type-specific expression, regulation, cellular localization, and function (Mancl et al, 2005). Human pDCs express four distinct alternatively spliced isoforms (V1, V2, V3, and V4) with V3 and V4 being the most predominant transcripts expressed in unstimulated pDCs. In contrast, the V4 (and also V1) isoforms are not detected in unstimulated T cells (Mancl et al, 2005). In addition, cell-type specific post-transcriptional regulation, for example by the expression of cell-specific microRNAs, may be involved in differences in IRF5 expression (Kriegel et al, 2013; Taniguchi et al, 2015). Basal IRF5 expression among pDCs is heterogeneous with some pDCs expressing no or low levels of IRF5. Although the antibody against IRF5 used in this study is recognizing all IRF5 isoforms, it is possible that differences in the affinity for the various isoforms may account for the low IRF5 levels reported in some pDCs. Overall, our data show that the role of IRF5 in IFN $\alpha$  transcription is cell-type-specific and resulting in differential sex-based expression of IRF5 as well as IFN $\alpha$  expression in pDCs.

In order to clarify the impact of IRF5 on IFN $\alpha$  transcription in primary human pDCs, a novel technique described by Sharei and colleagues to deliver exogenous recombinant protein into human primary pDCs was utilized (Sharei et al, 2013b). The delivery of recombinant IRF5 protein led to an increase in the percentage of IFN $\alpha$ -secreting pDCs confirming the *ex vivo* association observed between IRF5 levels and TLR7-induced IFN $\alpha$  production. In addition to IFN $\alpha$  production, IRF5 is also involved in the transcription of pro-inflammatory cytokines such as IL-6 and TNF $\alpha$  (Takaoka et al, 2005). In agreement with this, we observed an increase in the production of TNF $\alpha$ , IL-8, MIP-1 $\alpha$  and MIP-1 $\beta$  upon IRF5 delivery. Although Seillet and colleagues recently reported sex differences in TLR7-mediated TNF $\alpha$  production (Seillet et al, 2012), our group and others did not detect such difference (Berghofer et al, 2006; Meier et al, 2009). Interestingly, Seillet and colleagues used shorter stimulation times, suggesting that cytokine-specific feedback regulatory mechanisms may explain why sex difference in basal levels of IRF5 did not drive sex difference in the production of TNF $\alpha$  and other cytokines following TLR7 stimulation. One mechanism might come from a negative regulatory role of testosterone on the

production of TNF $\alpha$ , MIP-1 $\alpha$  and MIP-1 $\beta$ , as recently suggested (Bobjer et al, 2013). While the impact of sex differences in IRF5 basal levels on the production of pro-inflammatory cytokines may be limited by additional regulatory mechanisms, its impact on the sex difference in IFN $\alpha$  production was significant.

Biological sex differences in the human immune responses to infections or autoimmune diseases can be due to genetic factors linked to sex chromosomes, and/or the modulation of immunity by sex hormones. The precise functional mechanisms by which sex hormones might regulate the IFN $\alpha$  response of pDCs are unknown, but are thought to involve ER $\alpha$  signaling (Seillet et al, 2012; Seillet et al, 2013). ERs are expressed on all PBMCs including pDCs (Laffont et al, 2014; Mao et al, 2005; Phiel et al, 2005; Seillet et al, 2012; Yakimchuk et al, 2013). The interaction of ER $\alpha$  with target gene promoters can occur either directly, through specific estrogen response elements (EREs), or indirectly through contacts with other DNA bound transcription factors such as the specificity protein 1 (Sp1) but also as the activator protein 1 (AP1) or the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), both involved in the transcription of type I IFNs and proinflammatory cytokines (Bjornstrom & Sjoberg, 2005; Kushner et al, 2000; Safe & Kim, 2008; Stein & Yang, 1995). ER $\alpha$  may also regulate the IFN $\alpha$  production via transcriptional control of interferon regulatory factors. A previous study in mice showed higher IRF5 mRNA levels in splenic B cells from female than from age-matched male mice and lower levels of IRF5 mRNA in ER $\alpha$  knock-out mice as compared to wild-type mice (Shen et al, 2010). Interestingly, an ER $\alpha$  binding site has been identified using the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu>) at position 128561334-128561609 on human chromosome 7 which is 16,385bp upstream the *IRF5* gene (*IRF5* chr7:128577994-128590088), suggesting that IRF5 may be regulated by sex hormones at the transcriptional level through ER $\alpha$ . Furthermore, one of the described *IRF5* polymorphism, the CGGGG indel, is associated with increased expression of IRF5 itself due to the presence of an additional SP1 binding site (Clark et al, 2013; Sigurdsson et al, 2008). pDCs from Tie2-ER $\alpha$ <sup>KO</sup> and CD11c-ER $\alpha$ <sup>KO</sup> intact female mice lack ER $\alpha$  in the hematopoietic compartment or in CD11c<sup>+</sup> DC lineage, respectively, and have been shown to be impaired in their capacity to produce IFN $\alpha$  in response to TLR9 engagement (Seillet et al, 2012). We further show here that pDCs from Tie2-ER $\alpha$ <sup>KO</sup> and CD11c-ER $\alpha$ <sup>KO</sup> mouse are also altered in their ability to produce

IFN $\alpha$  following *ex vivo* TLR7 stimulation and that this was associated with decreased *IRF5* mRNA expression levels as compared to their littermate controls. Spliced variants of *IRF5* were identified only in human cells, while in inbred strains of mice *IRF5* encodes for a dominant unspliced transcript (Mancl et al, 2005). Such species-specific differences in splice patterns between *IRF5* may bias the translation of our results from one species to another. Nevertheless, we found a significant correlation between *IRF5* and *Esr1* mRNA levels in pDCs from females but not in pDCs from males in humans, highlighting the dependency of *IRF5* mRNA on estrogen signaling in females. Although we were not able to simultaneously measure *IRF5* mRNA and ER $\alpha$  protein in the same cells, previous reports suggested that *Esr1* mRNA and ER $\alpha$  protein expression correlate (Du et al, 2013; Pentheroudakis et al, 2013; Wirapati et al, 2008). The ER $\alpha$ <sup>ko</sup> mice models used in this study do not exclude that non-hormonal pathways and particularly X-chromosome linked factors participate in sex-specific regulation of *IRF5* in pDCs. Recently, it was shown that X chromosome dosage contributed independently from sex hormones to the sex bias in the pDCs TLR7-mediated IFN $\alpha$  response (Laffont et al, 2014). In line with these data, we observe a trend towards higher *IRF5* levels persisting in post-menopausal females as compared to age-matched males ( $p=0.06$ ). This might also explain why there were no difference in *IRF5* levels between pre-menopausal females under hormonal birth control and pre-menopausal females having regular menstrual cycle.

In conclusion, this study demonstrates that pDCs derived from healthy females exhibit higher basal levels of *IRF5* than pDCs derived from healthy males and that higher levels of *IRF5* are directly associated with higher IFN $\alpha$  responses to TLR7 stimulation. Furthermore, this sex-based difference appeared to be partially due to ER $\alpha$  signaling-mediated modulation, as *IRF5* mRNA expression was significantly reduced in mice with a conditional knock-out for ER $\alpha$  and correlated, in humans, to *Esr1* mRNA expression in pDCs from females. These data provide new insights into the mechanisms underlying the higher inflammation observed in females in infectious and autoimmune diseases, and identify *IRF5* as an attractive target for specific modulation of the IFN $\alpha$  pathway.

## MATERIAL AND METHODS

### Study subjects and samples

All human samples used were from individuals recruited and enrolled at Massachusetts General Hospital and all subjects gave written informed consent for participation in these studies. The study was approved by the Partners Human Research Committee (PHRC). Characteristics of the patient cohort are available in Table EVI. No significant differences in age ( $p=0.18$ , two-tailed Mann-Whitney test) nor ethnicity were noticed ( $p=0.1$ , Fischer Exact test) between the 53 females and 37 males included in this study. When available ( $n=26$ ), information on the use of oral contraceptives containing sex hormones was collected. The female study subjects included 18.9% ( $n = 10$ ) of postmenopausal or surgically sterile females. 50% ( $n = 8$ ) and 18% ( $n = 3$ ) of premenopausal females reported using oral contraceptives and using an intrauterine device, respectively. Subgroups were used in the different analysis performed, with some donors being tested across multiple assays. Blood was collected in sodium heparin tubes and PBMCs were separated from whole blood by Ficoll-Histopaque density centrifugation (Sigma-Aldrich, St Louis, MO). Cells were resuspended in R-10 [RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2500U/mL Penicillin, 2500 $\mu$ g/mL Streptomycin, 100mM L-Glutamine (Corning, Lowell, MA)] and counted. Blood was processed within 5 hours after venipuncture in order to prevent the loss of pDC responsiveness to TLR ligands (Meier et al, 2008).

### Mice

Mice selectively lacking ER $\alpha$  in the hematopoietic compartment or in the DC lineage were generated by crossing B6 mice carrying an *Esr1* gene in which exon 2 was flanked by loxP sites (ER $\alpha^{fl/fl}$ ) with B6 mice expressing the Cre recombinase under the control of the Tie2 promoter-enhancer (Tie2-ER $\alpha^{KO}$ ) or the CD11c promoter (CD11c-ER $\alpha^{KO}$ ) as described elsewhere (Lelu et al, 2011). Littermate wild type (WT) mice were used as controls. Mice were bred and maintained in a specific pathogen-free animal facility. 8-to-12-week-old female mice were used in all experiments. The INSERM U1043 institutional review board for animal experimentation approved protocols.

### **Measurement of single-cell cytokine production by flow cytometry**

Intracellular cytokine staining assays were carried out as previously described (Meier et al, 2009). Briefly, freshly isolated PBMCs were resuspended in R-10 at a concentration of 1.5 million cells/ml, and 1 ml of PBMCs were stimulated in FACS tubes with 1 µg/ml of CL097, a synthetic TLR7 ligand (Imidazoquinoline; Invivogen, San Diego CA). 5 µg/ml Brefeldin A (Sigma-Aldrich) was added to each tube immediately after addition of the stimulant in order to inhibit cellular cytokine release. Unstimulated cells with 5 µg/ml Brefeldin A added served as a negative control. Intracellular cytokine content of pDCs was determined following 20 hours of stimulation as previously described (Meier et al, 2007). PBMCs were stained for surface markers using anti-CD3 Alexa Fluor 700, anti-CD19 Alexa Fluor 700, anti-CD56 Alexa Fluor700, anti-CD11c PE, anti-CD14 APC-Cy7, anti-HLA-DR Pacific Blue and anti-CD123 PE-Cy5 (all from BD Biosciences, San Jose, CA). pDCs were defined as CD3<sup>neg</sup>CD19<sup>neg</sup>CD56<sup>neg</sup>HLA-DR<sup>pos</sup>CD14<sup>neg</sup>CD11c<sup>neg</sup>CD123<sup>bright</sup> cells. Cells were fixed and permeabilized using Fix&Perm Medium A and B (Invitrogen, Carlsbad, CA) and stained intracellularly with anti-IFNα FITC (PBL Interferon source, Piscataway, NJ), anti-IL-12 APC, and anti-TNFα PE-Cy7 (BD Biosciences). Flow cytometry data were acquired within two hours of staining on a BD Biosciences LSRII equipped with 4 lasers. Spectral overlap was corrected by appropriate compensation and rainbow beads were used to maintain the consistency of the fluorescence intensity between experiments. The frequency of cytokine-producing pDCs was determined by subsequent analysis using FlowJo software (version 8.5.2, Treestar, Inc, Ashland, OR). Unstimulated cells were used to define background cytokine production level and subtracted from the frequency in stimulated samples.

### **Measurement of *ex vivo* protein levels of transcription factors by flow cytometry**

Freshly isolated PBMCs were fixed by adding cold 4% paraformaldehyde (PFA) directly into the culture medium to obtain a final concentration of 2% PFA. Cells were incubated in fixative for 30 min at 37°C, then washed and pelleted. Cells were permeabilized by vortexing to resuspend in 500 µl ice-cold methanol (MeOH) and incubated for 10 min at -20°C, and subsequently washed twice. For IRF5 staining, fixed and permeabilized

cells were incubated for 10 min at room temperature and in the dark with the unconjugated IRF5 antibody (Abcam, Cambridge, MA), washed, and then stained with a secondary goat anti-rabbit Alexa Fluor 700 antibody (Invitrogen). Unconjugated rabbit IgG (Cell signaling Technology, Danvers, MA) was used as isotype control. Cells were stained for surface markers for 30 min at room temperature as described above. For IRF7 staining, anti-IRF7 Alexa Fluor 488 (BD Biosciences) was also added to the surface stain mix. Finally, the cells were washed, pelleted and resuspended in 100  $\mu$ L PBS containing 2% heat-inactivated FBS. All washes were performed with PBS containing 2% heat-inactivated FBS at 4°C. The IFN $\alpha$  secretion assay (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used in combination with this protocol as per manufacturer's instructions. Samples were acquired on the BD Biosciences LSRII within 2 hours of staining. The mean fluorescence intensities (MFI) of IRF5 and IRF7 in pDCs and CD3<sup>+</sup> T cells and the frequencies of IFN $\alpha$ -secreting pDCs were determined by subsequent analysis using FlowJo software.

### **Subcellular quantification of IRF5 protein levels in pDCs using the TissueFAXS slide scanning system**

Two million fresh PBMCs were seeded in 50  $\mu$ L R-10 on poly-d-lysine coated plates and simultaneously stimulated with 1  $\mu$ g/ $\mu$ L CL097 for 2 hours. Cells were then fixed with 4% PFA for 20 min at room temperature and permeabilized with ice-cold MeOH (10 min at -20°C). Unconjugated anti-IRF5 (Abcam) or rabbit IgG (Cell Signaling Technology) was added and the plates were incubated at 4°C overnight. The slides were subsequently washed in PBS supplemented with 2% normal goat serum and incubated in goat anti-rabbit IgG Alexa Fluor 546 (Invitrogen) for 30 min at room temperature. Cells were successively stained at room temperature with CD123 APC (BD Biosciences) for 1 hour, goat anti-mouse IgG2a Alexa Fluor 647 (Invitrogen) for 30 min, HLA-DR Alexa Fluor 488 for 1 hour (Exbio, Czech Republic), goat anti-mouse IgG1 Alexa Fluor 488 (Invitrogen) for 30 min, with three washes in PBS supplemented with 2% normal goat serum between each stain. All slides were mounted in Prolong Gold Antifade reagent with DAPI (Invitrogen). The sample slides were scanned using the TissueFAXS (TissueGnostics GmbH, Vienna, Austria) slide scanning system based on a Zeiss Axio Imager Z2 upright epifluorescence microscope. Images were captured using a Zeiss EC Plan-Neofluar 100x 1.3NA objective in combination with a PCO monochrome 12 bit CCD camera. This slide scanning system utilizes a Maerzhaeuser

motorized scanning stage with eight-slide holder to permit scanning and stitching together of many fields of view into one image. In this way, all of the plated cells could be scanned on each coverslip and then the fluorescence intensity of the different markers evaluated on a per-cell basis using TissueQuest imaging analysis software (TissueGnostics GmbH). pDCs were identified as HLA-DR<sup>+</sup>CD123<sup>+</sup> cells. Each fluorescence channel was thresholded in order to visually segment the cells based on average per-cell fluorescence intensity. Cytoplasm and nuclear IRF5 fluorescence was then separately determined using a cytoplasm mask for IRF5, a nuclear mask for IRF5, and a whole-cell mask for IRF5. For each sample, a minimum of 150 pDCs was imaged to determine the mean intensity of IRF5.

### **IRF5 recombinant protein delivery using a vector-free microfluidic platform**

pDCs were enriched from PBMCs using the pDC Enrichment Kit (Stemcell, Vancouver, Canada) following manufacturer's instructions. Cells were resuspended in R-10 at 1,000 cells/ $\mu$ L and were mixed with Cascade Blue-labeled 3-kDa dextran molecules, for control of delivery, and with either 0.03-0.06  $\mu$ g/ $\mu$ L of IRF5 recombinant protein (Abcam or Origene, Rockville, MD) or 0.05-0.1  $\mu$ g/ $\mu$ L of the control TUBA1A recombinant protein (Abcam), and subsequently placed in the device's inlet reservoir. Delivery was performed using a vector-free microfluidic platform as previously described (Sharei et al, 2013a; Sharei et al, 2013b) and illustrated in Figure EV2A. In brief, cells were mechanically deformed while passing through the microfluidic device (SQZ Biotechnologies, USA) at a pressure of 80 or 120psi, resulting in the transient formation of holes in the cell membrane allowing content from the surrounding buffer to diffuse into the cytosol. Cells were incubated at room temperature in the delivery solution for 5 min after treatment to ensure closure of membrane holes before being subjected to any further treatment, as previously described (Sharei et al, 2013a). Delivery efficiency was assessed using FITC-labeled 70 kDa dextran probes and/or Cascade Blue-labeled 3 kDa dextran molecules mimicking protein and siRNA deliveries respectively. Appropriate controls were included to correct for reduced IFN $\alpha$  producing capacity of pDCs subjected to mechanical deformation. Delivery of TUBA1A protein, which is not involved in the IFN $\alpha$  production pathway, was used as control for determining any non-specific effect on IFN $\alpha$  production. IRF5 levels were subsequently measured by flow cytometry as described above. Cells

were then stimulated for 2 hours with 1  $\mu\text{g}/\mu\text{L}$  CL097. Supernatants were collected and run on the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit (Millipore, Billerica, MA). Viability was assessed by using the Aqua LIVE/DEAD staining (Invitrogen) as per manufacturer's instructions. IFN $\alpha$  secretion was measured using the Miltenyi's IFN $\alpha$  secretion assay following manufacturer's instructions.

### **Quantification of *IRF5* mRNA expression in mouse pDCs**

Bone marrow (BM) cell suspensions were stained with APC-labeled mPDCA-1 and PE-Cy7-labeled anti-CD11c (all from eBiosciences) for 30 min at 4°C and double positive cells were sorted using a FACS-ARIA (BD Biosciences). RNA from purified pDCs was extracted using the NucleoSpin® RNA XS and treated with DNase I following manufacturer's instructions (Macherey-Nagel). RNA samples were retro-transcribed into cDNA using oligo-dT, random primers and the SuperScript® III Reverse Transcriptase (Life technologies). Quantitative PCR were performed using IRF5 QuantiTect Primer Assays with SYBR green PCR Mastermix (QIAGEN). Gene transcripts were normalized to HPRT gene abundance, and relative mRNA levels were calculated by the expression  $2^{-\Delta\text{Ct}} \times 100$ .

### ***In situ* IRF5 mRNA expression assay by flow cytometry**

Five million PBMCs were pelleted and surface stained on ice for 30 min. Cells were subjected to the QuantiGene FlowRNA assay (eBiosciences, San Diego, CA) as per manufacturer's instructions with type6-*B2M* probe, type1-*Esr1* probe and a customized ultrasensitive type4-*IRF5* probe (probes are all from eBiosciences). In order to control for non-specific probe interaction, type4-*IRF5* probe and type1-*Esr1* probe were replaced by type4-*TLR7* probe and type1-*TLR9* probe. The bacterial *DapB* probes were used as a control. In order to gain sensitivity, target incubation time was increased from 2 hours to 3 hours. Similarly, pre-amplification and amplification incubation times were increased from 1.5 hours to 2 hours. Samples were run in duplicates and acquired on the BD Biosciences Fortessa within 2 hours of staining. The MFI of *IRF5*, *Esr1* and *B2M* probes were determined by subsequent analysis using FlowJo software. Values were excluded if the duplicates exhibit more

than 20% difference.

### **Statistical analysis**

Comparison between sex was calculated with the Wilcoxon Rank tests (Mann-Whitney). Comparison of IRF5 MFI between IFN $\alpha$ -secreting pDC and non-secreting pDCs was calculated with the paired Wilcoxon Rank tests. Linear regression was calculated with Spearman rank-based correlation. For IRF5 protein delivery experiments, we used Wilcoxon signed rank for comparison of the increase in the percentage of IFN $\alpha$  secretion relative to the control therefore normalized to 1. Comparison between WT mice and ER $\alpha$ <sup>KO</sup> mice was calculated using the unpaired t tests.

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## **AUTHORSHIP CONTRIBUTIONS**

M.G., S.Z., S.L, L.C., N.S., G.K., C.D.P. and A.C.V. performed research; M.G., P.T., C.B, H.H, C.C and M.S. collected data; M.G. and J.C. analyzed data; A.S., F.P., M.H., O.S., J.P.H. and T.D. contributed vital new reagents or analytical tools; M.G., J.C.G., J.C. and M.A. designed the research; and M.G., S.L.G., B.A., J.C. and M.A. wrote the paper.

## **CONFLICT OF INTEREST DISCLOSURES**

A.S. has personal financial interest in SQZ Biotechnologies. AS had no influence on the results and discussion presented in this paper. M.G. was funded by eBiosciences to present her work using the FlowRNA® QuantiGene Assay at the meeting organized by the French Society of Immunology in Lille on November 5<sup>th</sup> 2014 (Conference fees, second class rail travel from Paris to Lille, 2 nights accommodation). S.Z. received a grant from Gilead to study the impact of pregnancy on the IFN $\alpha$  pathway in HIV-1-infected women.

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EXPANDED VIEW

**Table EVI. Characteristics of the cohort (healthy donors)**

<b>All cohorts</b>		<b>Females</b>	<b>Males</b>	<b>p-value</b>
		N= 53	N= 37	
<b>Age (Min-Max)</b>		32.7 <sup>#</sup> (15-82)	35.7 <sup>#</sup> (19-63)	0.20
<b>Ethnicity:</b>	White	40	27	1.0**
	Hispanic	1	2	
	Asian	5	3	
	Black/ African American	3	0	
	Hawaiian/Pacific	1	3	
	More than one race	1	0	
	Unknown	2	2	
<b>Use of hormonal contraceptives:</b>		N= 26		
	Postmenopausal	10		
	Premenopausal	16		
	Birth Control Pills	8		
	No contraceptive used	5		
	IntraUterine Device (IUD)	2		
	IUD with hormones	1		
<b>Cytokine Production (First cohort)</b>				
		N= 17	N= 14	
<b>Age (Min-Max)</b>		25.8 <sup>#</sup> (20-41)	36.7 <sup>#</sup> (22-58)	0.0041*
<b>Ethnicity:</b>	White	13	11	1.0**
	Others	3	3	
<b>IRF5 expression (Second cohort)</b>				
		N= 29	N= 19	
<b>Age (Min-Max)</b>		39 <sup>#</sup> (21-82)	34.5 <sup>#</sup> (20-63)	0.49*
<b>Ethnicity:</b>	White	20	14	1.0**
	Others	9	5	
<b>Donors part of the two cohort</b>				
		N= 6	N= 1	

<sup>#</sup>Mean

\*two-tailed Mann-Whitney t test

\*\* Fischer Exact test

**Table EVII: Description of the microfluidic devices tested**

SQUEEZING DEVICE: AA – (B) x C	AA	B	C
10-4iS	10	4	1
30-4iS	30	4	1
10-4x5iS	10	4	5

**AA:** length of constriction ( $\mu\text{m}$ )

**B:** width of constriction ( $\mu\text{m}$ )

**C:** number of times the constriction is repeated through each channel

## **SUPPLEMENTAL METHODS**

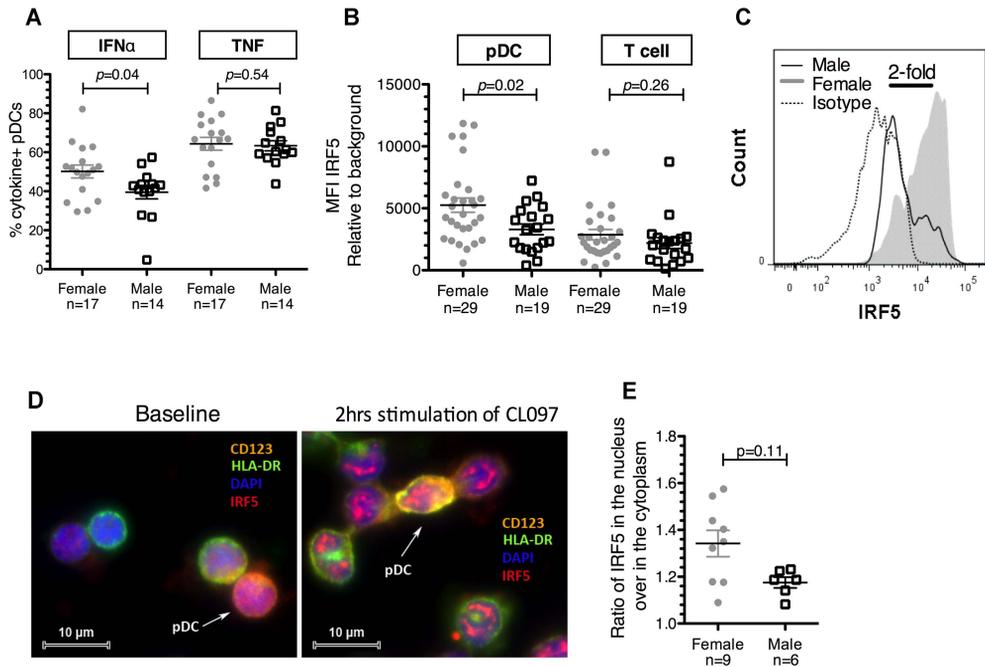
### **Intracellular cytokine staining of murine pDCs**

Murine bone marrow (BM) cell suspensions were activated with a preparation of oligonucleotides PolyU (Sigma-Aldrich) with 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP) (8  $\mu$ l of a cationic liposome preparation (DOTAP; Roche) mixed with 1  $\mu$ g of PolyU in 150  $\mu$ l of RPMI, in a polystyrene tube). BM cells were stimulated PolyU-DOTAP preparation for 4 hours and 5 $\mu$ g/ml brefeldin A (eBiosciences) was added for the last 2 h of culture. Non-specific staining was blocked with 5 $\mu$ g/ml anti-CD16/CD32 (2.4G2, American Type Culture Collection). BM cell suspensions were then stained with PE-Cy7-labeled anti-CD11c (N418) and APC-labeled mPDCA1 (all from eBiosciences). Intracellular cytokine staining was performed with mixed FITC-labeled IFN $\alpha$ / $\beta$ -specific antibodies (RMMA-1/RMMB-1; PBL). Unstimulated cells and isotypes were used as control staining. Data were acquired on a Fortessa (BD Biosciences).

### **Quantification of IRF5 protein levels in cytoplasmic fractions from pDCs by Western Blot**

pDCs were isolated with a purity >95% from fresh buffy coats using the pDC Diamond II kit (Miltenyi) as per manufacturer's instructions. Cytoplasmic proteins were extracted from pDCs using the NE-PER extraction kit (Pierce) as per manufacturer's instructions. Protein fractions from 5 females and 5 males were combined by sex and concentrated by vacuum before being fractionated by SDS 4-12% polyacrylamide gel electrophoresis (PAGE). Proteins were transferred by electroblotting onto polyvinylidene fluoride (PVDF) membranes (Immobilion). Membranes were probed for IRF5 (Abcam, ab124792) at 1:250 dilution,  $\beta$ actin (Cell signaling Technology, 3700S, clone 8H10D10) at 1:2000 dilution, HDAC1 (Cell signaling Technology) at 1:1000 dilution and HSP90 (Cell signaling Technology) at 1:1000 dilution. Secondary donkey anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680 (LiCor) were used at 1:5,000 dilution. IRF5 expression was measured using the Odyssey scanner (Licor) and quantified with the LiCor ImageStudio software. IRF5 expression was normalized to  $\beta$ actin.

# Figure 1



**Figure 1 - Sex differences in TLR7 signaling in pDCs**

(A) The percentage of interferon alpha (IFN $\alpha$ )-secreting plasmacytoid dendritic cells (pDCs) was significantly higher in females (n=17) than in males (n=14) while the percentage of tumor necrosis factor alpha (TNF $\alpha$ )-secreting pDCs did not differ between females and males after 20 hours of stimulation with 1  $\mu$ g/ml CL097. Brefeldin A was added at 5  $\mu$ g/ml at time of stimulation to block cytokine secretion.

(B) Mean fluorescent intensity (MFI) of IRF5 was determined *ex vivo* in pDCs and T cells from females (n=29) or age-matched male donors (n=19) using FlowJo<sup>®</sup> software. pDCs derived from females exhibited significantly higher interferon regulatory factor 5 (IRF5) levels than pDCs derived from males. IRF5 levels in T cells did not differ between females and males.

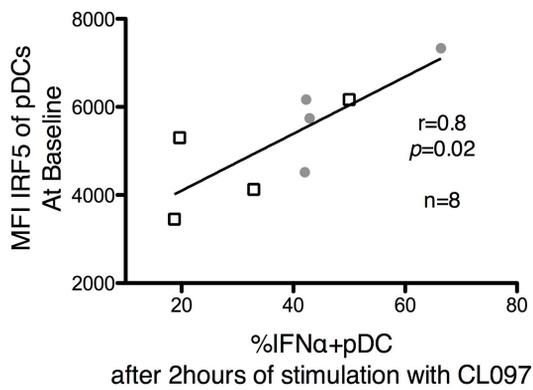
(C) Flow cytometry histogram overlays show the MFI of the IRF5 in pDCs from a male representative of the male average (empty curve) and in pDCs from a female representative of the female average (filled curve). There was a 2-fold difference in the MFI from those two samples. Isotype control is represented with dotted curve.

(D, E) PBMCs were stimulated with CL097 at 1  $\mu$ g/ $\mu$ L for 2 hours then stained for IRF5-Alexa Fluor 546, HLA-DR-Alexa Fluor 488 and CD123-Alexa Fluor 647. The sample slides were scanned in Prolong Gold using the slide scanning system based on a Zeiss Axio Imager Z2 upright epifluorescence microscope. Images were captured using a Zeiss EC Plan-Neofluar 100x 1.3NA objective in combination with a PCO monochrome 12 bit CCD camera. pDCs were identified among all of the plated cells as HLA-DR<sup>+</sup>CD123<sup>+</sup> cells. Quantification was performed using the TissueQuest analysis software.

(D) Representative images at baseline and after 2 hours of stimulation are shown with CD123 in orange, HLA-DR in green, DAPI staining of the nucleus in blue and IRF5 in red.

(E) The dot plot represents the ratio of nuclear IRF5 on cytoplasmic IRF5 in pDCs after 2 hours of stimulation with CL097. There was a trend towards higher IRF5 ratio in the nucleus in females (n=7) than in males (n=5). Error bars indicate the mean and SEM. Comparison between sexes was calculated using the Wilcoxon Rank tests.

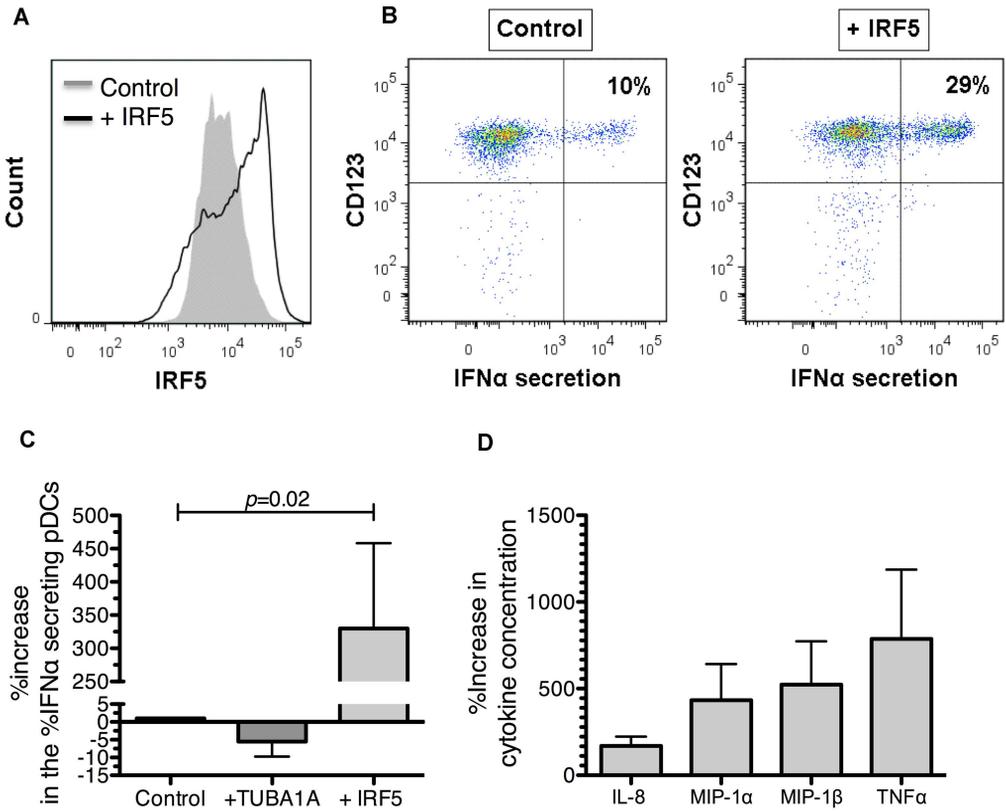
## Figure 2



**Figure 2 - IRF5 levels in pDCs are associated with IFN $\alpha$  secretion upon TLR stimulation**

Interferon regulatory factor 5 (IRF5) and interferon alpha (IFN $\alpha$ ) expression were measured in plasmacytoid dendritic cells (pDCs) after 2 hours of stimulation with CL097. A significant correlation between the basal levels of IRF5 in pDCs and the percentage of IFN $\alpha$ -secreting pDCs after 2 hours of stimulation with CL097 was observed. Linear regression was calculated in 8 healthy donors (females  $n=4$ , close round shapes; males  $n=4$ , open squares) with Spearman rank-based correlation.

# Figure 3



**Figure 3 - Exogenous IRF5 enhance IFN $\alpha$  production after TLR7 stimulation**

Enriched plasmacytoid dendritic cells (pDCs) population was subjected to the microfluidic device with or without (control) recombinant IRF5 protein in the surrounding milieu. IRF5 levels were measured at baseline. pDCs were then stimulated with CL097 for 2 hours and IFN $\alpha$  secretion was measured.

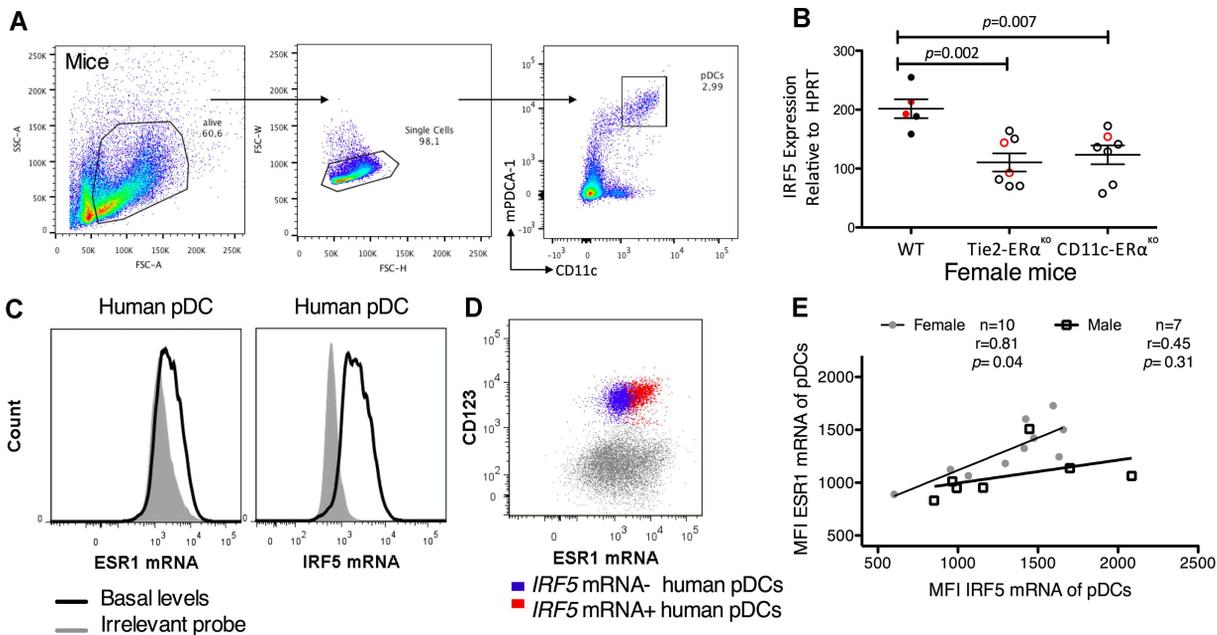
(A) Flow cytometry histogram overlays show the mean intensities of the IRF5 in pDCs following IRF5 delivery (empty curve) and in the control (filled curve) after treatment with the microfluidic device. A two-fold increase in the MFI of IRF5 in pDCs is observed upon IRF5 delivery.

(B) Primary flow data of IFN $\alpha$  secretion in pDCs after 2 hours of TLR7 stimulation in the control sample and after IRF5 delivery

(C) The percentage of IFN $\alpha$ -secreting pDCs increased specifically upon delivery of IRF5 protein. The percentage of IFN $\alpha$ -secreting pDCs upon delivery of IRF5 was normalized to the control. In contrast, no significant changes occurred in the percentage of IFN $\alpha$ -secreting pDCs upon delivery of TUBA1A, a protein irrelevant to the IFN $\alpha$  pathway. Data for IRF5 delivery are representative of 7 independent experiments. Wilcoxon signed rank for comparison of the percentage increase in the percentage of IFN $\alpha$  secretion relative to the control therefore normalized to 1.

(D) Supernatants were collected after 2 hours of stimulation and run on the Milliplex Human 29 cytokine/chemokine magnetic bead panel kit (Millipore). Increase in the concentration of TNF $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$  and IL-8 is observed upon delivery of IRF5 protein.

# Figure 4



**Figure 4 - Sex difference in IRF5 levels in pDCs is associated to estrogen signaling**

(A, B) pDCs from the bone marrow of female wild-type (WT) (n=12), Tie2-ER $\alpha$ <sup>KO</sup> (n=14) and CD11c-ER $\alpha$ <sup>KO</sup> (n=10) mice were cell sorted based on the expression of CD11c and PDCA-1 from bone marrow cells.

(A) Gating strategy for pDC sorting is shown

(B) RNA was isolated for each group from 1-2 pools of 4-5 mice (in red) and from 3-6 individual mice (in black). IRF5 expression was quantified by qRT-PCR and normalized according to HPRT expression. IRF5 mRNA levels were significantly lower in pDCs from Tie2-ER $\alpha$ <sup>KO</sup> and CD11c-ER $\alpha$ <sup>KO</sup> than in pDCs from WT mice. Comparison between mice groups was calculated using the unpaired t tests

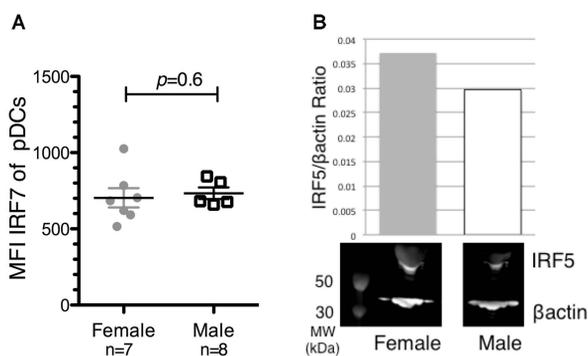
(C-E) mRNA levels of IRF5 and ESR1 were measured in human pDCs using the QuantiGene FlowRNA assay.

(C) Flow cytometry histograms overlays show the mean intensities of IRF5 or ESR1 mRNA (empty curve) compared to control with the irrelevant *dapB* probes (filled curve) in pDCs.

(D) IRF5 mRNA+ and IRF5 mRNA- pDC populations distribute into ESR1 mRNA+ and ESR1 mRNA- pDC population respectively.

(E) A significant correlation between IRF5 and ESR1 mRNA levels is observed in human pDCs derived from females (n=10,  $p=0.04$ ,  $r=0.84$ ) but not in pDCs derived from males (n=7,  $p=0.31$ ,  $r=0.45$ ). Samples were run in duplicates. Linear regression was calculated with Spearman rank-based correlation.

# Figure EV1

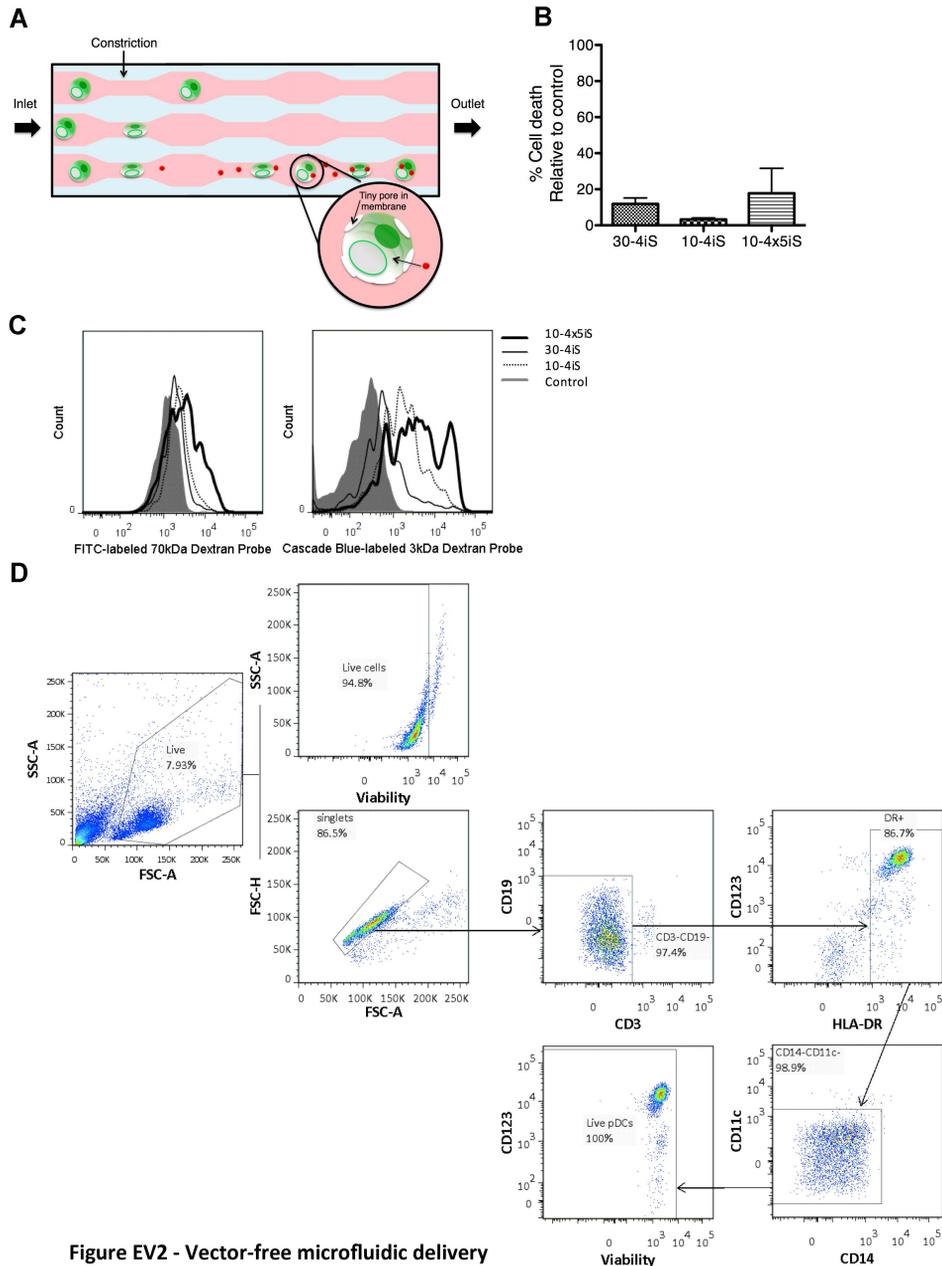


**Figure EV1 – IRF7 and IRF5 expression in pDCs in females compared to males**

(A) Median fluorescent intensity of interferon regulatory factor 7 (IRF7) was determined *ex vivo* in plasmacytoid dendritic cells (pDCs) from females (n=7) or age-matched male donors (n=5) using FlowJo software. No difference between sex in IRF7 expression in pDCs was observed. Comparison between sexes was calculated using the Wilcoxon Rank tests

(B) pDCs were isolated by magnetic separation with a purity >95% from fresh buffy coats. Cytoplasmic proteins were extracted from pDCs. Protein fractions from 5 females and 5 males were combined by sex and concentrated by vacuum before being analyzed by Western Blotting. Membranes were probed for IRF5 (Abcam, ab124792) and  $\beta$ actin (Cell signaling Technology, 3700S, clone 8H10D10). IRF5 expression was normalized to  $\beta$ actin. Higher IRF5 expression relative to  $\beta$ actin was observed in the cytoplasmic fractions from females than from males.

# Figure EV2



**Figure EV2 - Vector-free microfluidic delivery**

(A) The principle of the delivery system is schematized. Cells (green) and delivery material (blue and red) are mixed in the inlet reservoir. By passing through the microfluidic device, cells are deformed so that transient holes are generated allowing for delivery of the surroundings material. Cells are collected in the outlet reservoir.

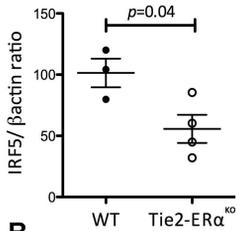
(B) The delivery technique did not significantly impact pDCs' survival. Column bar graph shows the percentage of cell death relative to the control for endocytosis uptake. These data are representative of two independent experiments. Error bars represent SEM.

(C) Different microfluidic devices were tested for delivery of protein to human primary pDCs. A population enriched in pDCs was treated with different microfluidic devices at 80 psi to deliver 3-kDa (*right*) and 70-kDa (*left*) dextran molecules, used to mimic siRNA delivery and protein delivery respectively. Untreated cells were put in contact with the dextran molecules for control for endocytosis uptake (Control). Microfluidic devices are classified by their constriction dimension (AA – (B) x C) where AA and B are the constriction length ( $\mu\text{m}$ ) and width ( $\mu\text{m}$ ), respectively, and C the number of constrictions in series per channel. Efficiencies and viabilities were measured by flow cytometry after staining with the aqua LIVE/DEAD viability within the hour. The histogram overlays show the mean intensities of the 70- and 3-kDa probes in pDCs following treatment with different microfluidic devices (colored lines) and corresponding untreated negative controls (grey filled curves).

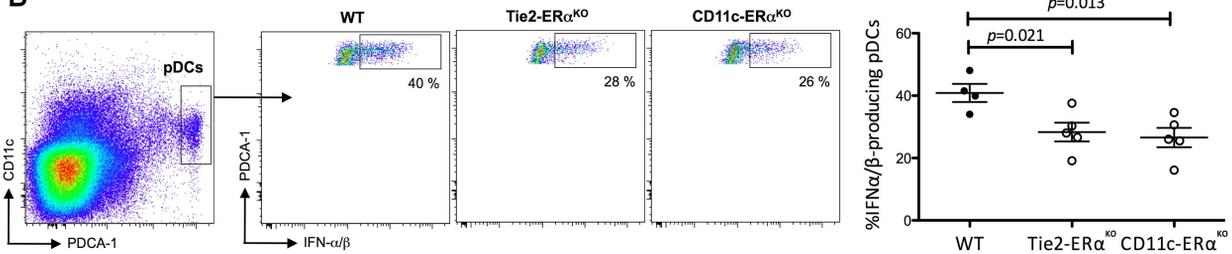
(D) Gating strategy of pDCs after passing through microfluidic device is shown. Only live pDCs are observed after standardized sequential gating strategy as demonstrated by co-staining with viability dye.

# Figure EV3

## A Splenic B cells



## B



**Figure EV3 – ERα deletion impacts IRF5 protein levels in splenic B cells and is associated with diminished TLR7-mediated production of IFNα by pDCs**

(A) B cells were individually purified from wild-type (WT) (n=3) and Tie2-ERα<sup>KO</sup> (n=4) mice spleens by positive selection using anti-CD19 beads (Miltenyi). Protein lysates of B cells were analyzed by immunoblotting for IRF5 and βactin protein expression. Densitometry results normalized to βactin show decreased IRF5 protein levels in splenic B cells from Tie2-ERα<sup>KO</sup> as compared to WT mice.

(B,C) Bone marrow cells from WT, Tie2-ERα<sup>KO</sup> and CD11c-ERα<sup>KO</sup> mice were stimulated with the TLR7 ligand PolyU for 4 hours, in presence of brefeldin A for the last 2 hours.

(B) Representative flow cytometry plots showing IFNα production by pDC (CD11c<sup>+</sup>PDCA1<sup>+</sup>).

(C) ERα deletion in hematopoietic compartment (Tie2-ERα<sup>KO</sup>) or in the DC lineage (CD11c-ERα<sup>KO</sup>) was associated with diminished frequency of IFNα/β-producing pDCs from mice upon TLR7 stimulation. Comparison between mice groups was calculated using the unpaired t tests.



## Study n°2

### **HCV drives increased inflammation and pDC impairments associated to disease severity in ART-treated HIV-1-HCV co-infected individuals**

Morgane Griesbeck, Christine Blanc, Julie Bottero, Marc-Antoine Valantin, Michèle Pauchard, Karine Lacombe, Catherine Blanc, Amandine Emarre, Zineb Sbihi, Aurélien Corneau, Gloria Martrus, Véronique Morin, Anne Oudin, Rima Zoorob, Assia Samri, Marguerite Guiguet, J. Judy Chang, Marcus Altfeld and Brigitte Autran

In preparation

Immune activation levels have been compared between HCV-HIV-1 co-infected and HIV-1 mono-infected individuals in several studies. Many described increased immune activation levels in HCV-HIV-1 co-infection<sup>412,413,415</sup>. Those findings prompted the design of our HepACT-VIH study. As pDCs sense both HIV-1 and HCV via TLR7, we hypothesized that alterations of the pDC TLR7/IFN $\alpha$  pathway may account for the increased immune activation observed. Small numbers of patients were recruited in the aforementioned studies, with very similar inclusion numbers to our protocol (HepACT-VIH). However, our strict inclusion criteria, while limiting the potential application of our findings, also diminished confounding factors. In particular, our HCV-HIV-1 co-infected and HIV-1 mono-infected patients were very well matched based on the characteristics of HIV-1 disease. Furthermore, the levels of cellular immune activation were evaluated on fresh whole blood, limiting bias due to thawing-freezing cycle. Last but not least, we performed a comprehensive analysis, evaluating soluble markers of inflammation, activation of the TLR7/IFN $\alpha$  pathway and transcriptomic analysis, together with the levels of cellular immune activation. In that regard, the study presented here is original.

We found that inflammation but not cellular immune activation is greater in HCV-HIV-1 co-infected individuals than in HIV-1 mono-infected individuals. We identified a stronger ISGs signature in pDCs from HCV-HIV-1 co-infected individuals, which was associated to disease severity. Inflammation is associated with accelerated fibrosis progression and the development of co-morbidities in ART-treated HIV-1 infected individuals. Current treatment guidelines recommend treating only patients with severe fibrosis. In light of emergent DAA treatments, with poor toxicity and good tolerability profiles, our results suggest that HCV-HIV-1 co-infected patients could benefit from earlier treatment.

## **HCV drives increased inflammation and pDC impairment associated to fibrosis severity in ART-treated HIV-1-HCV co-infected individuals**

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## Abstract

**Background:** Increased immune activation and inflammation in HIV-1-HCV co-infected patients may be driving increased disease progression in the post-ART era. We investigated whether such impairments are associated to alterations in the pDC IFN $\alpha$ /TLR7 pathway in HIV-1-HCV-co-infected.

**Methods:** Cellular activation, plasma levels of inflammatory cytokines and pDC transcriptome associated to IFN $\alpha$ /TLR7 pathway was extensively characterized in 15 HIV-1-HCV-co-infected individuals with mild to moderate and 14 HIV-1-mono-infected individuals with similar HIV-1 disease history. Healthy donors' samples were tested as controls.

**Results:** HIV-1-HCV-co-infected individuals exhibited higher inflammation (IP-10, MIP-1 $\beta$ , IL-8 and ITAC) than HIV-1-mono-infected patients ( $p=0.0007$ ,  $p=0.028$ ,  $p=0.028$  and  $p=0.035$  respectively). pDCs and T cells from HIV-1-HCV-co-infected individuals displayed a more exhausted (defined by LAG-3 and CD57 expression respectively) but not activated phenotype. pDC IFN $\alpha$ /TLR7 pathway was constitutively activated in HIV-1-infected patients, irrespective of HCV co-infection and despite suppressive ART. Increased expression of the interferon stimulated genes (ISGs) E12AK2, ISG15, Mx1 and IFI44 was observed in pDCs from HIV-1-HCV-co-infected individuals and was correlated with fibrosis score ( $p=0.02$ ).

**Conclusion:** HCV drives increased inflammation and ISGs expression in pDCs, associated to fibrosis severity in ART-treated HIV-1-infected patients with mild to moderate fibrosis.

## INTRODUCTION

Immune activation and inflammation play a deleterious in infection by the human immunodeficiency virus type 1 (HIV-1). They predict disease progression in untreated infection [1-4] but also contribute to increased risk of serious non-AIDS-related morbidity and mortality, such as cardiovascular and liver diseases [5-9], in treated patients. When compared with uninfected controls, elevated immune activation and inflammation persist in HIV-1-infected individuals on antiretroviral treatment (ART) even when HIV-1 viremia is fully suppressed [5, 10-14]. Immune activation and inflammation levels can be enhanced directly by HIV-1 replication and indirectly through loss of gut tissue integrity and microbial products translocation [15], persistent production of type I interferons (IFN) [16-18], co-infection with pathogens [19], immune deregulation and lymphoid tissue fibrosis [20-23].

About 30% of HIV-1-infected individuals in Europe and United States of America are co-infected by HCV [24-26]. Accelerated hepatic fibrosis progression, higher rates of liver failure, and death have been reported in HIV-1-HCV-co-infection compared to HCV-mono-infection [27-31]. HIV-1 infection negatively impacts every stage of HCV infection: enhancing HCV transmission, decreasing HCV clearance rates [32] and to higher HCV viral load (VL) [33]. A recent meta-analysis of over 30 studies gathering a total of 100,000 patients showed that in the post-ART era, co-infection increased of about 35% the risk of overall mortality but not of AIDS-defining conditions compared to HIV-1-mono-infection [34]. High levels of chemokines including IP-10, ITAC, MIP-1 $\beta$  and IL-8 have been found in the blood of HCV-infected patients with healthy controls [35-37]. Whether increased morbidity stems directly from HCV replication or is the result of collateral damage from long-standing inflammation remain unclear [38]. In both cases,

co-infection by HIV-1 and HCV is likely to increase immune activation and inflammation as suggested by some studies [22, 39]. The emergence of HCV direct-acting antiviral (DAA) therapy agents have led to tremendous progresses in the treatment of HCV in terms of both efficacy and toxicity profiles [40-43]. However, increased levels of immune activation and inflammation and their persistence duration may still affect the development of co-morbidities.

Plasmacytoid dendritic cells (pDCs) are the professional type I IFNs producers. pDCs-derived IFN $\alpha$  is involved in several aspects of HIV-1 immunopathogenesis, including enhancement of T cell activation markers [17]. During both HIV-1- and HCV-mono-infections and HIV-1-HCV-co-infection, there is a loss of circulating pDCs in the periphery [44-48]. Both HIV-1 and HCV single-stranded RNAs activate pDCs via TLR7 [49-51]. Genetic polymorphisms in TLR7, which is encoded on the X chromosome, have been associated with differential disease outcome in both HCV- and HIV-1-infections [52, 53]. Both HIV-1 and HCV impair pDC-associated IFN $\alpha$ /TLR7 pathway [54, 55] but the pDCs IFN $\alpha$  response resulting from dual infections is not easily predictable. Nevertheless, one study on a very small number of patients reported higher IFN $\alpha$  plasma levels in co-infected versus HIV-1-mono-infected individuals [56]. Stronger alterations in the pDC IFN $\alpha$ /TLR7 pathway in HCV-HIV-1-co-infected patients may therefore shape immune activation and inflammation in those patients.

In this study, we show that HCV drives increased inflammation but not immune activation in HIV-1-infected patients under suppressive ART. pDC IFN $\alpha$ /TLR7 pathway is constitutively activated in HIV-1-infected patients, irrespective of HCV-co-infection and despite suppressive ART. Increased expression of ISGs in pDCs from co-infected individuals was associated with fibrosis score. Understanding whether HCV-co-infection affects HIV-1-induced immune activation and/or systemic inflammation in the context

of suppressive ART may elucidate challenging issues for providers treating HIV-1 patients with HCV-co-infection and shed insight into the complex interactions between these two persistent viruses.

## **MATERIAL AND METHODS**

### **Study subjects and samples**

We recruited 29 ART-treated chronically HIV-1-infected male patients followed at the Pitié-Salpêtrière and Saint-Antoine hospitals (Paris, France). Patients had plasma HIV-1 RNA level below 50 copies/mL and a CD4+ T cell count  $\geq 350$  cells/mm<sup>3</sup> for at least 6 months before inclusion. Patients with autoimmune or inflammatory diseases, hepatitis B co-infection, with active intravenous drug use and/or with present alcohol abuse were excluded. HIV-1-HCV-co-infected individuals had detectable HCV viral load, were infected by HCV genotype 1 and naïve for HCV treatment. Five additional HIV-1-HCV-co-infected patients were included based on similar criteria at the exception of the requirement for a severe fibrosis while inclusion criteria regarding HCV treatment was exempted. Patient characteristics were collected from medical records and from a survey. Liver fibrosis was assessed using transient elastography in HIV-1-HCV-co-infected individuals (Fibroscan, [www.echosens.com](http://www.echosens.com), Paris, France) and classified using the METAVIR scoring system with F0–F2 being mild to moderate fibrosis (cut-off 9.5 kPa) and METAVIR F3–F4 being severe fibrosis to cirrhosis (cut-off 9.5 kPa). Blood from anonymous healthy volunteers was requested from the bloodbank 'Etablissement français du sang'. The study was reviewed and approved by the ethics committee of Paris VI. Four HCV-HIV-1-co-infected patients from the first cohort received a 12-weeks IFN-free anti-HCV therapy and plasma samples were collected 12 weeks after treatment completion.

Blood was collected in sodium heparin tubes and processed within 5 hours. Plasma was collected and stored at -80°C. PBMCs were separated from whole blood by Ficoll density gradient and either used directly or cryopreserved.

### **Measurements of plasma levels of cytokines**

Plasma samples were run on the ProCarta Human Ultrasensitive 9 plex kit (IFN $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL12p70, IL-17A, TNF $\alpha$ ), the ProCarta simplex TGF- $\beta$ , a ProCarta customized 7-plex (IFN $\alpha$ , IL-8, IP-10, TNF $\alpha$ , I-TAC, TRAIL, sCD40L, MIP-1 $\beta$ ) (All from eBioscience). All IFN $\alpha$  subtypes were measured using the human IFN- $\alpha$  ELISAPRO kit (Mabtech, Sweden).

### **Measurement of PBMCs activation and absolute quantification by flow cytometry**

For T cell activation, 100 $\mu$ L whole blood were stained for surface markers using anti-CD3-Brilliant Violet (BV)711, anti-CD4-BV605, anti-HLA-DR-APC-Cy7, anti-CD25-BV421, anti-CD38-PE-Cy7, anti-CD57-PE, anti-CD69-FITC (all from BD Biosciences, San Jose, CA) and anti-CD8 Alexa Fluor (AF)700 (Beckman Coulter, Fullerton, CA). For antigen-presenting cells (APC) activation, 500 $\mu$ L whole blood were stained for surface markers using anti-CD14-Brilliant Ultraviolet (BUV)395, anti-CD3/CD56/CD19-AF700, anti-CD11c-APC, anti-CD123-PE-Cy5, anti-HLA-DR-BV421, anti-CD16-APC-Cy7, anti-CD163-PE-CF594, anti-CD40-PE, anti-CD80-PE-Cy7, anti-CD86-FITC (all from BD Biosciences) and anti-CCR7-BV711 (eBiosciences, San Diego, CA). Red blood cells were lysed and cells fixed using the BD Lysis Buffer. For T cell activation, cells were subsequently permeabilized (Perm Buffer B, Invitrogen, Carlsbad, CA) and simultaneously stained with anti-Ki67-AF647 (BD Biosciences). For absolute quantification of pDCs, CD4 $^{+}$  and CD8 $^{+}$  T cells, 500 $\mu$ L whole blood were introduced in Trucount tube and processed according to manufacturer's instructions. Flow cytometry samples were all acquired on the BD Biosciences Fortessa within 3 hours of staining. The percentage of activated CD4 $^{+}$  and CD8 $^{+}$  T cells, the mean fluorescence

intensities (MFI) of activation markers on pDCs, mDCs and monocytes subsets and absolute quantification were determined by subsequent analysis using FlowJo software.

### **Measurement of phospho-proteins involved in pDC TLR7/IFN $\alpha$ signaling by flow cytometry**

Freshly isolated PBMCs were stained using anti-CD3/19/56-BV421, anti-CD11c-PE, anti-CD14-PE-Cy7, CD123-PE-Cy5 (all from BD Biosciences) and anti-HLA-DR-eFluor605NC (eBiosciences). Paraformaldehyde-fixed cells were permeabilized by methanol and stained with anti-pIRF7-AF488 and anti-pSTAT1-AF647 (BD Biosciences). The MFI of pSTAT1 and pIRF7 in pDCs were determined using FlowJo software.

### *Cell sorting*

Freshly thawed PBMCs were stained for surface markers using anti-CD3 PerCP-Cy5.5, anti-CD19 Alexa Fluor 700, anti-CD56 Alexa Fluor 700, anti-CD11c Alexa Fluor 700, anti-CD14 Alexa Fluor 700, anti-HLA-DR APC-Cy7, anti-CD8 PE-Cy7, anti-CD57 PE, anti-CD123 PE-Cy5 (all from BD Biosciences, San Jose, CA), anti-CD303 APC (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), anti-LAG3 FITC (RnD Systems, Minneapolis, MN) and the viability dye Zombie Red (eBioscience). pDCs, defined among live cells as CD3<sup>neg</sup>CD56<sup>neg</sup>CD19<sup>neg</sup>CD14<sup>neg</sup>CD11c<sup>neg</sup>HLA-DR<sup>pos</sup>CD123<sup>bright</sup>CD303<sup>pos</sup> cells, were sorted using a FACS-Aria™ Fusion (BD Biosciences). One million events were collected for analysis of LAG-3 expression on pDCs. One hundred pDCs were collected in biological duplicates for all patients but for one HIV-1-HCV co-infected individual with severe fibrosis. Cells were sorted into a 96 PCR plate preloaded with 5uL lysis buffer [1x VILO Reaction mix (Life technologies), 6U SUPERase-in (Life technologies), 10% Nonidet P-40 (Thermo Fisher)]. The plates were centrifuged for 1 min at 4°C and immediately frozen for one hour.

### *Primer Design and validation*

The 96 Genes evaluated were for the overwhelming majority part of the type I IFNs/TLR7 pathway in pDCs. The mRNA sequences of the studied genes were taken from the National Center for Biotechnology Information (NCBI) Gene database (NCBI, National Library of Medicine, Bethesda, MD, USA). Primer pair oligos were designed by DeltaGene (Fluidigm). Specificity of primer pairs was checked via melting curve analysis and PCR efficiencies of the assays were determined with a 6-point dilution series of cDNA from FLU-stimulated Gen2.2 cells (pDC-like cell line) and/or primary pDCs from healthy donor stimulated with CL097 for 16 hours. Primer sequences and gene names are shown in Supplementary Table S6.

### *cDNA preparation*

cDNA was prepared by adding 0.25X SuperScript Enzyme Mix (Invitrogen) and 0.12 $\mu$ L T4 Gene 32 Protein (New England Biolabs, Ipswich, MA) to the 5 $\mu$ L of lysed cells for a final volume reaction of 6 $\mu$ L. Reverse transcription was performed by sequential steps : 5min at 25°C, 30min at 50°C, 25min at 55°C, 5min at 60°C, followed by a last step of 10min at 70°C. cDNA was stored at -20°C. For preamplification of cDNA samples, the PreAmp Master Mix (Fluidigm, South San Francisco, CA) was used according to the manufacturer's instructions. Primers were used a 50nM. 1.25  $\mu$ L of cDNA in a 5  $\mu$ L reaction was preamplified with the thermal protocol : 95°C for 2min, followed by 18 cycles of 95°C for 15sec and 60°C for 4 min. Samples were then treated with the exonuclease I (New England Biolabs) to remove unincorporated primers. Samples were then diluted in 1:5 in TE buffer and stored at -20°C.

### *qPCR*

5 $\mu$ L sample premix consisting of 2.5 $\mu$ L SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.25  $\mu$ L 20X

DNA Binding Dye Sample loading reagent (Fluidigm) and 2.25  $\mu$ L preamplified and prediluted cDNA, as well as 5 $\mu$ L assay premix consisting of 0.25 $\mu$ L 100 $\mu$ M primer pairs, 2.5 $\mu$ L Assay loading reagent (Fluidigm) and 2.25 $\mu$ L of TE buffer were prepared and transferred to the primed GE Dynamic Array 96.96. The samples and assays were mixed inside the chip using the IFC controller (Fluidigm). qPCR of the 83 preamplified and pre-diluted cDNA samples from 42 patients together with a no template control and internal control from FLU-stimulated Gen2.2 cells was conducted on the BioMark™ HD system. Thermocycling parameters included a thermal mix step at 70°C for 40min followed by 30sec at 60°C, a hot start at 95°C for 60sec followed by 30 PCR cycles consisting 96°C for 5sec and 60°C for 20sec. After completion of the run, a melting curve of the amplified products was determined.

#### *Data preprocessing and analysis*

Real-time PCR Analysis Software (Fluidigm) was used to determine the valid PCR reactions. Invalid reactions were not used for later analyses and treated as missing data. Raw Cq values were processed with Genomics Suite (Partek, Saint-Louis, MO) using Partek default settings. Outliers were excluded based on principal component analysis (PCA) and particularly based on the expression of the housekeeping gene PTMA, three data points of the HIV-1-HCV co-infected individuals (two being biological duplicates from the same patient), one data point from the HIV-1 mono-infected individuals and two data points from HIV-1-HCV co-infected individuals with severe fibrosis (including the biological monuplicate) appeared to be significant outliers and were removed from further analysis, leaving 85 remaining data points for comparison, corresponding to 14 HCV-HIV-1 co-infected, 14 HIV-1 mono-infected individuals, 9 healthy controls and 3 HIV-1-HCV co-infected individuals with severe fibrosis. The reference gene PTMA, stable across all patient groups, was subtracted to target gene Cq. The mean

between biological duplicates was calculated. Differentially expressed genes (comparing various HIV-1 infected patients groups to healthy controls) were selected at more than 2-fold difference and  $p$ -value with false discovery rate (FDR)  $<0.05$ .

### **Transcriptomic analysis of PBMCs using the BioMark™ (Fluidigm)**

RNA from 100,000 PBMCs was extracted using the RNeasy Mini kit (Qiagen, Netherlands). DNase treatment was performed on column following manufacturer's instructions. cDNA was prepared from 50ng RNA using the Reverse transcriptase core kit (Eurogentec, Liège, Belgium). cDNA samples was pre-amplified as described above with 12 primers (*STAT1*, *Mx1*, *EI2AK2*, *ISG15*, *IFI44*, *IFNAR1*, *TP53*, *NLRP3*, *PTMA*, *GNAS*, *IFIH1* and *PRDM1*). Samples were treated with the exonuclease I and diluted in 1:5 in TE buffer and stored at  $-20^{\circ}\text{C}$ . qPCR was performed as described above with the following changes. Premix was done in FLEXsix™ DELTAgene Sample Reagent instead of 20X DNA Binding Dye Sample loading reagent (Fluidigm). 3uL of sample premix and Assay premix were transferred to the primed FlexSix IFC. Data preprocessing and analysis were performed as described above.

### **Statistical analysis**

Comparison between two groups was calculated with the Wilcoxon Rank tests (Mann-Whitney). Linear regression was calculated with Spearman rank-based correlation. Transcriptomic analysis was performed using Partek Genomics Suite as described above.

## RESULTS

### Patient characteristics

We enrolled 42 male subjects, including 15 HIV-1-HCV genotype 1-co-infected patients naïve for any anti-HCV drugs, 14 HIV-1-mono-infected patients and 13 uninfected controls. Characteristics of the different groups are shown in Table 1. All HIV-1 infected patients (both mono-infected and HCV-co-infected) were on suppressive ART with 350 CD4 cells/mm<sup>3</sup> and HIV-1 viral loads below 20 copies/mL for all patients but one (54 copies/mL at time of inclusion with previous and subsequent values below 20 copies/mL). The HIV-1-mono-infected and the HIV-1-HCV-co-infected groups were well matched for HIV-1 disease characteristics including HIV-1 duration, ART duration, CD4 T cell nadir. The median HCV viral load were of 204,7170 copies/mL. Due to the criteria on the absence of any previous anti-HCV treatment, the HIV-1-HCV-co-infected patients recruited had mainly mild fibrosis (F0-F1) with one patient with F2 score. As expected, the ALAT and ASAT values were lower in HIV-1-mono-infected than in HIV-1-HCV co-infected patients ( $p=0.001$  and  $p=0.0037$  respectively).

### HCV drives increased inflammation in ART-treated HIV-1-infected patients

First, we assessed the levels of inflammation markers IFN $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL12p70, IL-17A, TNF $\alpha$ , TGF- $\beta$ , IL-8, IP-10, TNF $\alpha$ , I-TAC, TRAIL, sCD40L, MIP-1 $\beta$  and IFN $\alpha$  in our patient groups. Plasma levels of IP-10, IL-8, MIP-1 $\beta$  and ITAC were significantly higher in HIV-1-HCV-co-infected than in HIV-1-mono-infected patients, after correction for multiple tests (Benjamini, Hochberg, 1995) (Figure 2, Supplementary Table 1). A trend towards higher IFN $\alpha$  levels in HIV-1-HCV-co-infected than in HIV-1-mono-infected individuals was observed ( $p=0.06$ , Figure 1, Supplementary Table 1). Out of the 16

parameters measured, only IP-10 in HCV-HIV-1-co-infected individuals and IFN $\alpha$  in HIV-1-infected individuals, irrespective of HCV status, were significantly higher than in healthy donors after correction for multiple tests (Benjamini, Hochberg, 1995) (Figure 1, Supplementary Table 1). Besides, IL-1 $\beta$ , IL12p70, IL-2, IL-17A and IL-4 were not detected in any samples.

Increased inflammation could be related to hepatic fibrosis but none of these parameters were correlated to fibrosis severity. As our cohort only included patients with minimal fibrosis, we recruited five additional HCV-HIV-1-co-infected individuals with severe fibrosis. Characteristics of these patients are described in Supplementary Table 2. Cytokines levels in HCV-HIV-1-co-infected individuals were independent of fibrosis stages. Taken together, these data suggest that HCV replication may be driving increased inflammation.

### **HCV-HIV-1 co-infection triggers exhaustion but not activation of peripheral blood pDCs and T cells**

We then analyzed the activation levels of pDCs and other APCs. Levels of CD40, CD86, CCR7 and/or CD163 on pDCs, conventional DC (cDCs) or monocytes subsets were influenced neither by the HIV-1 nor by the HCV status (Figure 2A and Supplemental Figure 1). Nevertheless, we observed a trend toward higher CD40 expression on CD14<sup>++</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup> monocytes in HCV-HIV-1-co-infected compared to HIV-1-mono-infected individuals (Supplemental Figure 2B-C,  $p=0.08$  and  $p=0.07$  respectively). We analyzed pDCs exhaustion using the lymphocyte activation gene 3 (LAG-3), an exhaustion marker expressed by a subset of pDCs, which negatively regulates T cell activation and positively regulates Treg function [57, 58]. The expression of LAG-3, although low, was significantly

higher in pDCs from HCV-HIV-1-co-infected than from HIV-1-mono-infected individuals (Figure 2B,  $p=0.04$ ).

We next evaluated the activation levels of CD4+ T cells and CD8+ T cells as determined by the expression of CD25, CD69, CD38 and HLA-DR (Figure 2C, Supplemental Figure 2, Supplemental Table 3). Expression of CD25 and CD69 did not vary across patient groups (data not shown). In contrast, the co-expression of CD38 and HLADR on CD4+ and CD8+ T cells, while not increased by HCV-co-infection ( $p=0.62$  and  $p=0.53$  for CD4+ and CD8+ T cells respectively), was significantly higher in ART-treated HIV-1-infected individuals compared to healthy donors ( $p=0.048$  and  $p=0.016$  for HCV-HIV-1-co-infected and HIV-1-mono-infected individuals respectively). Similarly, expression of CD38 alone was not influenced by either HIV-1 or HCV status (data not shown). Given our observation of increased exhaustion of pDCs, we measured the expression of the immunosenescent marker CD57 on T cells. HCV co-infection influenced only the percentage of CD8+CD57+ cells, with a significantly higher proportion in co-infected (Figure 2D,  $p=0.014$ ) whereas the percentages of both CD57-expressing CD4+ and CD8+ T cells were significantly higher in HIV-1-infected individuals than in healthy donors ( $p=0.0055$  and  $p=0.0001$  respectively). None of the percentages of CD38+HLADR+ or CD57+ CD8+ T cells were correlated to HCV viral load in HCV-HIV-1-co-infected individuals ( $p=0.12$ ,  $R^2=0.18$ ;  $p=0.12$ ,  $R^2=0.012$ , respectively). As expected, the percentage of CD57+CD8+ was negatively correlated to the CD4/CD8 ratios in HIV-1-infected individuals ( $p=0.018$ ,  $R^2=0.19$ ). No correlation was found between pDC LAG-3 expression and T cell activation. We also enumerated the absolute counts of pDCs in parallel of CD4+ and CD8+ T cells (Supplementary Figure 3A). Altogether, our results suggest that in a well-controlled ART-treated HIV-1 infection with minimal defects in the CD4+T cell compartment, HCV does not contribute to higher immune activation but is

associated with a generalized immune exhaustion.

### **Persistent impairment in IFN $\alpha$ pathway in pDCs in HIV-1-infected individuals under suppressive antiretroviral therapy**

We quantified *ex vivo* levels of phosphorylated STAT1 (pSTAT1) and IRF7 (pIRF7) in pDCs to assess the activation of the IFN $\alpha$  pathway. No difference was observed between HCV-HIV-1-co-infected individuals and HIV-1-mono-infected individuals. ART-treated HIV-1-infected individuals had significantly more pSTAT1 than healthy donors (Figure 3B-C,  $p=0.007$  and  $p=0.004$  for HCV-HIV-1-co-infected and HIV-1-mono-infected individuals respectively). A trend towards higher pIRF7 in HIV-1-infected individuals, compared to healthy donors was noticed (Figure 3C,  $p=0.11$  and  $p=0.09$  for HCV-HIV-1-co-infected and HIV-1-mono-infected individuals respectively). Altogether, these data suggest that despite suppressive ART, pDC IFN $\alpha$  pathway is persistently activated and may therefore contribute to immune dysfunction in well-treated HIV-1-infected individuals.

### **Strong type I IFNs signature in pDCs from HCV-HIV-1 co-infected individuals under suppressive ART**

To gain further insights into pDC IFN $\alpha$  pathway activation, we performed a transcriptomic analysis of 96 genes mainly related to type I IFN/TLR7 pathway in sorted pDCs, defined among live cells as CD3<sup>neg</sup>CD56<sup>neg</sup>CD19<sup>neg</sup>CD14<sup>neg</sup>CD11c<sup>neg</sup>HLA-DR<sup>pos</sup>CD123<sup>bright</sup>CD303<sup>pos</sup> cells, from all three study groups (15 HCV-HIV-1-co-infected, 14 HIV-1-mono-infected individuals and 9 healthy controls) and from 4 HCV-HIV-

1-co-infected individuals with severe fibrosis. A heatmap of the results is shown in Figure 4A. PCA allowed distinguishing healthy controls from HIV-1-infected individuals irrespective of their HCV status (Figure 4B). Differentially expressed genes (DEG) were defined by a  $p$ -value with FDR of  $<0.05$  and twofold or greater difference in relative expression between patient groups versus healthy controls. There were 8, 10 and 4 DEGs detected in HIV-1-HCV-co-infected individuals with minimal fibrosis, with severe fibrosis and in HIV-1-monoinfected individuals respectively as shown in a Venn diagram (Figure 4C). Four interferon-stimulated genes (*IFI44*, *EI2AK2/PKR*, *ISG15*, *Mx1*) and *STAT1* were upregulated in HIV-1-HCV-co-infected individuals, irrespective of fibrosis stage compared to healthy donors (Figure 4D). Upregulation was at least two-fold stronger in patients with severe fibrosis (Supplementary table 4). An “ISG score” was defined by the average expression of the four aforementioned ISGs. No upregulation of ISGs or *STAT1* was observed in HIV-1 mono-infected individuals compared to healthy controls (Figure 4D). To assess if ISGs were upregulated in all mononuclear cells, we measured the expression of the four ISGs in PBMCs. No differences were observed in the expression of any of those genes in PBMCs among the patients groups as illustrated for *Mx1* and *EI2AK2* in Supplementary Figure 4A-B, suggesting that ISGs signature is specific to the pDCs compartment. In addition, only one gene, the interferon induced with helicase C domain 1 (*IFIH1*) was at least 2-fold upregulated in all HIV-1-infected patient groups compared to healthy donors with an about 6-fold upregulation in HIV-1-HCV-co-infected individuals with severe fibrosis (Figure 4C, Supplementary table 4).

### **Impairments in HCV-HIV-1 co-infection correlate with disease severity**

Inflammatory markers were not correlated to fibrosis score (data not shown) but may be linked to HCV replication. We had access to plasma samples from four HCV-HIV-1-co-infected patients from the first cohort 12 weeks after completion of an IFN-free anti-HCV treatment and evaluate the impact of HCV clearance on the cytokine levels. As exemplified for IP-10 (Figure 1F), these preliminary results show a reduction in the levels of those cytokines. Taken together, these data suggest that HCV replication may be driving increased inflammation. Furthermore, pDC absolute numbers were significantly lower in HCV-HIV-1-co-infected individuals with severe fibrosis than in individuals with minimal fibrosis or healthy donors (Supplementary Figure 4B,  $p=0.009$  and  $p=0.008$ , respectively). Interestingly, the absolute counts of pDCs were correlated to the percentage of CD57+CD8+ T cells (Supplementary Figure 3C,  $p=0.026$ ,  $R^2=0.35$ ) but not to Fibroscan values (data not shown). The “ISG score” was correlated to parameters of the HCV disease severity (Figure 4E), including Fibroscan score ( $p=0.026$ ,  $R^2=0.35$ ), ALAT levels ( $p=0.018$ ,  $R^2=0.34$ ,) and APRI score ( $p=0.019$ ,  $R^2=0.4$ ,) in HIV-1-HCV-co-infected individuals with mild to severe fibrosis but not to HCV viral load (data not shown). pDCs STAT1 expression was also correlated to ALAT levels ( $p=0.0094$ ,  $R^2=0.37$ ) and Fibroscan score ( $p=0.0012$ ,  $R^2=0.60$ ) (Supplementary table 5).

## DISCUSSION

In our study, we found increased levels of inflammatory cytokines in HCV-HIV-1-co-infected individuals with mild to moderate fibrosis than in HIV-1-mono-infected patients, among which IP-10 was the most prominent and decreased at 12 weeks post-completion of IFN-free DAA treatment. In contrast, we did not observe any difference in the activation of T cells, DCs or monocytes. Increased exhaustion, defined by higher frequency of CD57+CD8+ T cells and increased LAG-3 expression on pDCs, was seen in HCV-HIV-1-co-infected. In addition, increased expression of ISGs in pDCs from HIV-1-HCV infected patients correlated with fibrosis score. Overall, our results suggest that, even in the context of minimal fibrosis, HCV-HIV-1-co-infected individuals suffer from increased immune impairments.

Biomarkers associated with mortality in ART-treated HIV-1-infected persons reflect predominantly chronic activation of the innate rather than adaptive immune system [59, 60]. In line with these data, we found higher levels of inflammatory cytokines, including IP-10, but similar levels cellular immune activation. Although inflammation can be driven by hepatic fibrosis, our results rather suggest that HCV replication is the primarily cause for inflammation in our study. Indeed, the patients studied had minimal fibrosis. Furthermore, IP-10 may be a surrogate marker for HCV replication [61]. Consistently, we observed a decrease in IP-10 levels upon HCV clearance. Methodological differences including inclusion criteria can partially explain discrepancies with previous studies reporting increased T cell activation in HCV-HIV-1-co-infection [22, 39, 62]. In our study, T cell activation was measured on fresh whole blood. Consistently, we reported lower percentage of CD38+HLADR+ CD8+ in all groups than in studies performed on PBMCs [62]. Our study only included male, HCV genotype 1-infected patients, naïve for any HCV treatment. In addition, HIV-1-mono-infected patients were matched to HCV-HIV-1-co-

infected individuals based on their CD4/CD8 ratio, which may have attenuated potential differences in T cell activation given that the CD4/CD8 ratio is associated to immune activation [63]. Nevertheless, increased T cell activation was seen in HIV-1-infected individuals compared to healthy controls, in accordance with previous reports suggesting that the levels of activation of T cells fail to reach normal levels even under suppressive ART [11-14]. Besides, we observed a generalized exhaustion phenotype in HCV-HIV-1-co-infection defined by increased LAG-3 expression on pDCs and higher frequency of CD57+CD8+ T cells, consistent with the expansion of CD57-expressing CD8+ lymphocytes upon chronic antigenic exposure. The percentage of CD57+CD8+ was negatively correlated to the CD4/CD8 ratio in HIV-1-infected individuals, in agreement with previous reports [63, 64]. Interestingly, the percentage of CD57+CD8+ correlated to the absolute count of pDCs in the peripheral blood in the HIV-1-HCV-co-infected individuals. The reduced pDC numbers observed in patients with severe fibrosis may be related to hepatic sequestration.

Last but not least, we found increased ISGs levels in pDCs from HIV-1-HCV-co-infected patients than in HIV-1-mono-infected patients. Importantly, pDCs ISGs levels correlated with three independent measures of fibrosis. Increased ISGs expression may reflect pDC *in vivo* refractoriness to HCV infection. However, HCV can prevent ISGs translation and therefore efficient antiviral responses [37, 65, 66]. Furthermore, ISGs expression was higher in patients with severe fibrosis than in patients with minimal fibrosis. We therefore hypothesized that the ISGs signature in HCV-HIV-1-co-infected patients can be seen as a surrogate marker for increased IFN $\alpha$  levels, although the difference in IFN $\alpha$  levels did not reach significance. Increased inflammation may also be associated to IFN $\alpha$  impairment as IP-10 is upregulated by type I IFNs. Importantly, no increase in ISGs expression in HCV-HIV-1-co-infection was

observed in PBMCs. This finding is unclear, as the upregulation of hundreds of ISGs has been reported in the peripheral blood of chronically HCV-infected patients [67]. Increased expression of ISGs and phosphorylation and nuclear localization of STAT1 in the liver of HCV infected patients have been associated to refractoriness to IFN $\alpha$  treatment [67]. Some studies have reported that pre-activation of the IFN pathway in PBMCs could similarly predict responsiveness to IFN therapy [68, 69]. Strong ISGs expression in these patients may therefore suggest unresponsiveness to IFN therapy, which is generally less efficient in co-infected patients than in HCV-mono-infected [70]. In the context of emergent IFN-free DAA treatments, clinical practice guidelines no longer differentiate between HCV-HIV-1-co-infected and HCV-mono-infected patients and recommend to treat only patients with significant fibrosis [71, 72]. Excessive inflammation is associated with the occurrence of non-AIDS defining events [60] and favors HCV-mediated liver damage [73, 74]. Our study provides a rationale for treating HCV-HIV-1-co-infected individuals with DAAs, even with mild fibrosis, as early as possible, and despite their high costs.

Our study has several limitations, including a recruitment strategy biased towards minimal fibrosis. In addition, fibrosis is a long process while ISGs expression is a much more time-sensitive measure. Then, our findings are restricted to male sex and HCV genotype 1. Moreover, we did not include a group of HCV-mono-infected patients. Finally, IFN $\lambda$  polymorphisms have been associated to ISGs expression levels [75] and to liver fibrosis [76]. Our study could have benefit from further investigation on type III IFN signaling.

Altogether, this study shows that HCV-HIV-1-co-infected individuals even with minimal fibrosis display systemic immune impairments associated to fibrosis severity. Altered pDC type I IFN signaling and HCV replication may drive such impairments. Earlier treatments should be considered in those

patients.

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## **AUTHORSHIP CONTRIBUTIONS**

M.G., C.B., M.P, Z.S., V.M. and R.Z. performed research; M.G., M.A.V, K.L., J.B., C.K., J.C., M.A. and B.A. designed the research;; M.G. and M.Gu. analyzed data; C.B. contributed vital new reagents or analytical tools; and M.G., and B.A. wrote the paper.

## **CONFLICT OF INTEREST DISCLOSURES**

The authors have no conflict of interest.

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## Tables

**Table 1: Study subjects characteristics**

Groups	HIV-1-mono-infected individuals	HIV-1-HCV-co-infected individuals	Healthy controls	p-value
	n=14	n=15	n=13	
<b>General characteristics</b>				
Gender	Male	Male	Male	
Age (year) (range)	51.73 (40-59)	47 (36.5-54)	38.3 (23-51)	<b>0.04</b>
Ethnicity			NA	0.32 &
Caucasian, number (%)	13 (93%)	11 (73%)		
African, number (%)	1 (7%)	1 (6.6%)		
Maghrebian, number (%)	0 (0%)	1 (6.6%)		
West Indian, number (%)	0 (0%)	1 (6.6%)		
Unknown, number (%)	0 (0%)	1 (6.6%)		
BMI $\geq$ 25 kg/m <sup>2</sup> (%)	5 (35.7%)	5 (33%)	NA	1 &
History of IDUs			NA	0.018 &
Active, nombre (%)	0 (0%)	0 (0%)		
Past, nombre (%)	0 (0%)	5 (33%)		
No, nombre (%)	14 (100%)	10 (66%)		
<b>HIV-1 disease characteristics</b>				
HIV-1 Plasma Viral Load (copies/mL)	<20	< 20 (Except 1 VL =54)	NA	-
CD4 absolute median (cells/uL) (range) *	605 (380-1033)	716.6 (400-1478)	NA	0.18
CD4 median % (range)*	34.3 (24-46)	32.8 (19-46)	NA	0.73
CD8 absolute median (cells/uL) (range) *	676 (442-1139)	922 (406-2262)	NA	0.21
CD8 median % (range)*	39.3 (25-56)	41.4 (26-63)	NA	0.68
Ratio CD4/CD8 median (range) *	0.76 (0.46-1.99)	1.03 (0.37-1.84)	NA	0.98
Nadir CD4				
<200, number (%), range	8 (57%), (1-195)	8 (53%), (3-171)		-
200-350, number (%)	5 (35.7%)	5 (33%)		-
>350, number (%)	1 (7%)	2 (13%)		-
Estimated duration of HIV-1 infection			NA	
Years (Range)	18.3 (6-27)	18.8 (4-28)		0.84
> 10 years (%)	13 (93%)	14 (93%)		-
ARV Therapy			NA	
Years of ART (range)	15.9 (3-26)	12.2 (3-23)		0.19
Years between diagnostic and HAART (range)	2.7 (0-9)	6.6 (0-21)	NA	0.32

<b>ART Regimen</b>				
PI-based regimen (%)	9 (64%)	8 (53%)	NA	0.71 &
Current use of atazanavir (%)	1 (7%)	3 (21%)		0.59
Former use of maraviroc (%)	1 (7%)	1 (7%)		1
<b>HCV Disease characteristics</b>				
<b>Estimated duration of HCV infection</b>				
Years (Range)	NA	12 (2-23)	NA	
> 10 years (%)	NA	9 (60%)	NA	
<b>HCV treatment</b>	NA		NA	
Naïve, number (%)		<b>15 (100%)</b>		
Previous IFN-based treatment, number (%)		0 (0%)		
<b>Median HCV VL (copies/mL)</b>	NA	2047170 (207026-5842054)	NA	-
<b>HCV genotype</b>				
% Genotype 1a	NA	10 (66%)	NA	-
% Genotype 1b	NA	5 (33%)	NA	-
% Unknown	NA	0 (0%)	NA	-
<b>Order of infection</b>		HIV-1 infection first		
<b>Liver fibrosis/cirrhosis</b>				
% F0-F1, number (%)	NA	14 (93%)	NA	
% F2, number (%)	NA	1 (7%) **	NA	
% F3-F4, number (%)	NA	0 (0%)	NA	
<b>Patients with Fibroscan score available within 6months, number (%)</b>	NA	11 (73%)	NA	
<b>Fibroscan score (range) #</b>	NA	6.5 (4.8-12.2)	NA	
<b>ALAT (IU/L) (range) *</b>	30 (8-105)	55 (39-92)	NA	<b>0.001</b>
Patients with elevated ALAT (%) \$\$	1 (7%)	1 (7%)	NA	
<b>ASAT (IU/L) (range) *</b>	29.8 (21 -49)	42 (28-61)	NA	<b>0.004</b>
Patients with elevated AST (%) \$\$\$	0 (0%)	1 (7%)	NA	
<b>IL28B genotype (rs12979860)</b>	NA		NA	
% Genotype C/C	4 (26.7%)			
% Genotype C/T	3 (20%)			
% Genotype T/T	2 (13.3%)			
% Unknown	6 (40%)			

NA : Not available, not applicable

\* Sample collection within 1 month of the inclusion (Sample from 1 HCV-HIV-1-coinfected individuals was collected 2.5 months before inclusion)

\*\* Inconsistent diagnostic between fibroscan and fibrotest

# For patients with fibroscan available within 6 months of the inclusion

\$\$ Patients with more than 2 times the upper normal limit (Normal ranges for ALAT : 11-40 UI/L)

\$\$\$ Patients with more than 2 times the upper normal limit (Normal ranges for ASAT : 10-30 UI/L)

Mann-Whitney

& Fisher Exact

## Figure Legend

### **Figure 1: Comparison of the plasma levels of inflammatory markers between HCV-HIV-1 co-infected, HIV-1 mono-infected treated individuals and healthy controls.**

Plasma levels of panIFN $\alpha$  (all subtypes) (A) were measured by ELISA while plasma levels of IP-10 (CXCL-10) (B), ITAC (CXCL11) (C), IL-8 (D) and MIP-1 $\beta$  (E) were measured by Luminex in 15 HCV-HIV-1 co-infected, 12 HIV-1 mono-infected treated individuals and 5 healthy donors (and 12 healthy donors for panIFN $\alpha$ ). IP-10 plasma levels were measured by Luminex for four HCV-HIV-1 co-infected patients at the time of inclusion (Baseline) and 12 weeks post-treatment completion (F). Comparison between groups was performed by two-tailed Mann-Whitney t test and comparison between baseline and post-treatment levels by two-tailed Wilcoxon matched-pairs signed rank test. Horizontal bars show median.

### **Figure 2: Cellular activation and exhaustion of pDCs and T cells in HCV-HIV-1 co-infected, HIV-1 mono-infected patients and healthy controls**

(A) Fresh whole blood was stained for T-cell costimulatory markers CD40 and CD86, the lymph node homing receptor CCR7 and the hemoglobin scavenger receptor CD163 and expression of markers of activation on pDCs from 14 HCV-HIV-1 co-infected, 14 HIV-1 mono-infected patients and 3 healthy controls.

(B) LAG-3 expression was measured on pDCs from frozen samples in 15 HCV-HIV-1 co-infected, 14 HIV-1 mono-infected patients and 10 healthy controls.

(C, D) Percentages of CD8+HLA-DR+CD38+ (C) and CD8+CD57+ (D) T cells are shown for 15 HIV-1-HCV co-infected individuals, 14 HIV-1 mono-infected individuals and 13 healthy controls.

Comparison between groups was performed by Mann-Whitney t test. Horizontal bars show median.

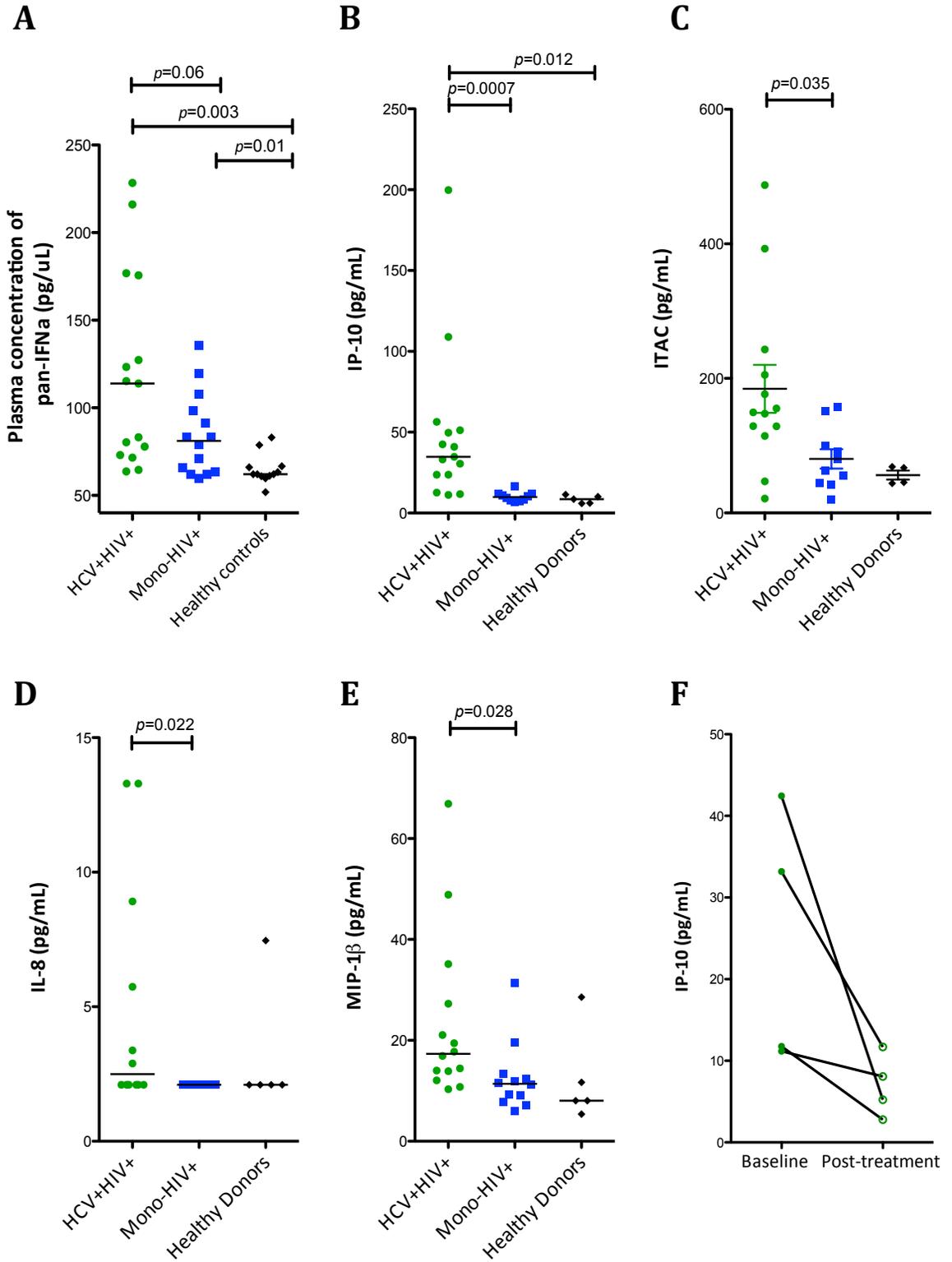
### **Figure 3: Persistent activation of IFN $\alpha$ signaling in pDCs in HIV-1 infected individuals despite suppressive ART, irrespective of HCV status.**

Gating strategy (A). Representative flow cytometry histograms of the expression of phosphorylated STAT1 in pDCs (B). Combined results for 15 HCV-HIV-1 coinfecting individuals under suppressive ART, 14 HIV-1 monoinfected individuals under suppressive ART and 5 healthy controls for the mean fluorescence intensity of phosphorylated STAT1 and IRF7 in pDCs (C). Comparison between groups was performed by Mann-Whitney t test. Horizontal bars show median.

**Figure 4: Transcriptomic analysis of pDCs from HCV-HIV-1 co-infected individuals with minimal to moderate fibrosis, severe fibrosis, HIV-1 mono-infected individuals and healthy donors**

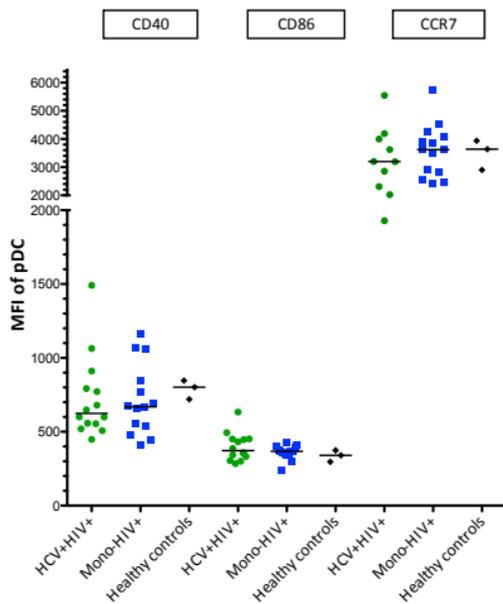
100 pDCs were sorted in biological duplicates for 15 HCV-HIV-1 co-infected, 14 HIV-1 mono-infected individuals, 9 healthy controls and 4 HIV-1-HCV co-infected individuals with severe fibrosis before being analyzed on the Fluidigm Biomark HD. A heatmap of the results is shown (A). After exclusion of outliers and normalization to the housekeeping gene PTMA, means between duplicates was calculated. Segregation between groups by principal component analysis is shown (B). Differentially expressed genes in 14 HCV-HIV-1 infected patients with minimal to moderate fibrosis (HCV+HCV+=<F2), 14 HIV-1 mono-infected patients (MonoHIV+) and in 4 HCV-HIV-1 infected patients severe fibrosis (HCV+HCV+>F2), with a *p*-value with false discovery rate of <0.05 and two-fold or greater difference in relative expression to 9 healthy controls (HC) (C). The relative expression of *IFI44*, *Mx1*, *ISG15*, *EI2AK2* and *STAT1* in pDCs is compared between the groups (D). Comparison was performed by Mann-Whitney t test with multiple tests correction (Benjamini et Hochberg, 1995). ISG score, defined as the average of *IFI44*, *Mx1*, *ISG15* and *EI2AK2* expressions in pDCs, correlated to Fibroscan values, ALAT levels, APRI score (E). Linear regression was calculated with Spearman rank-based correlation.

**Figure 1**

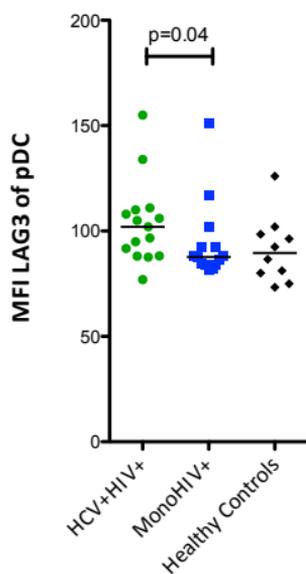


# Figure 2

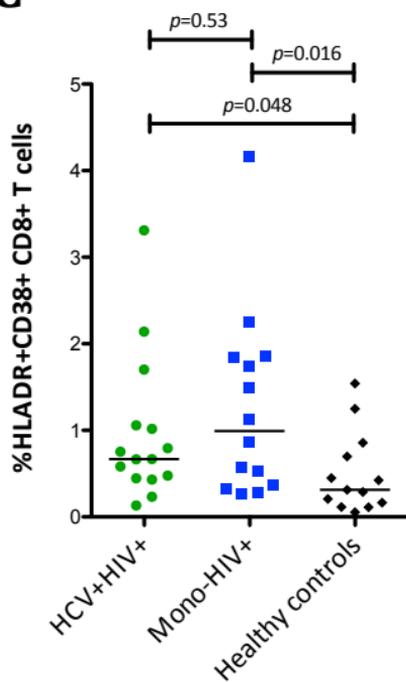
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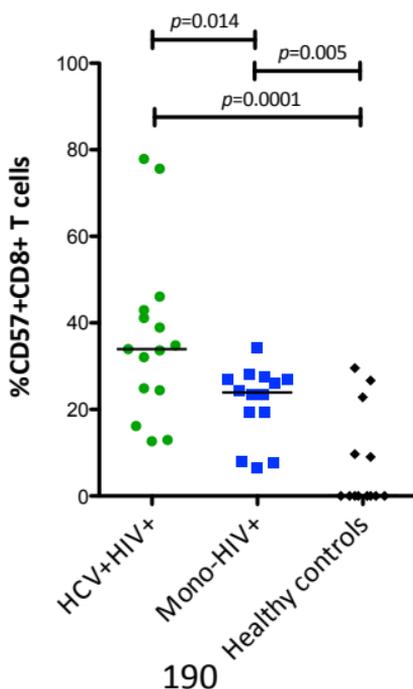
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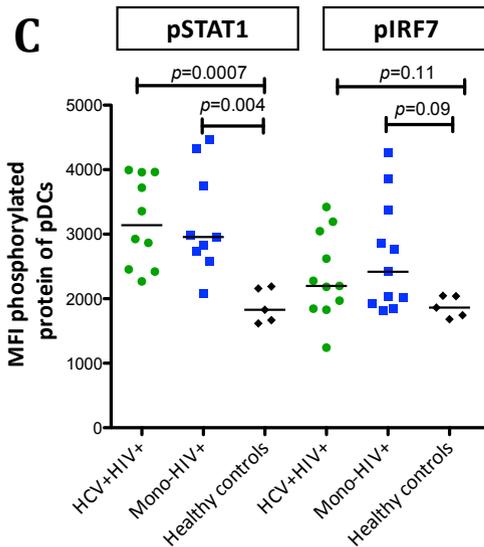
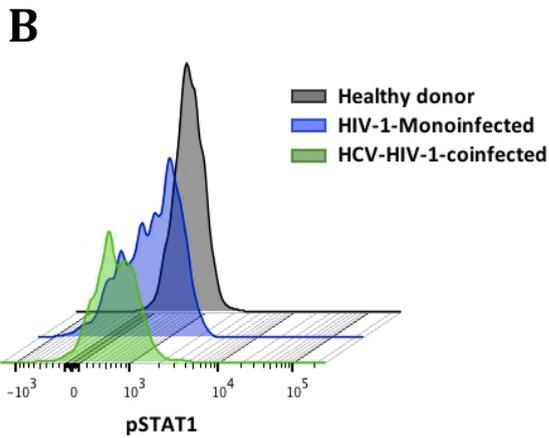
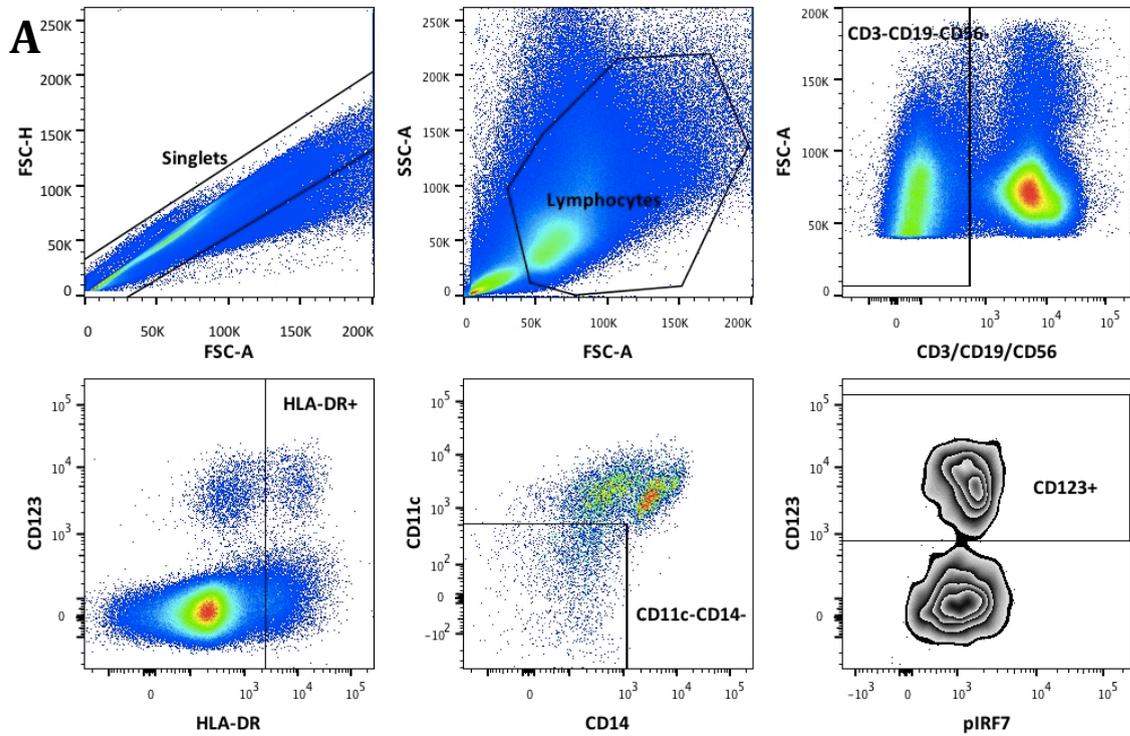
## C

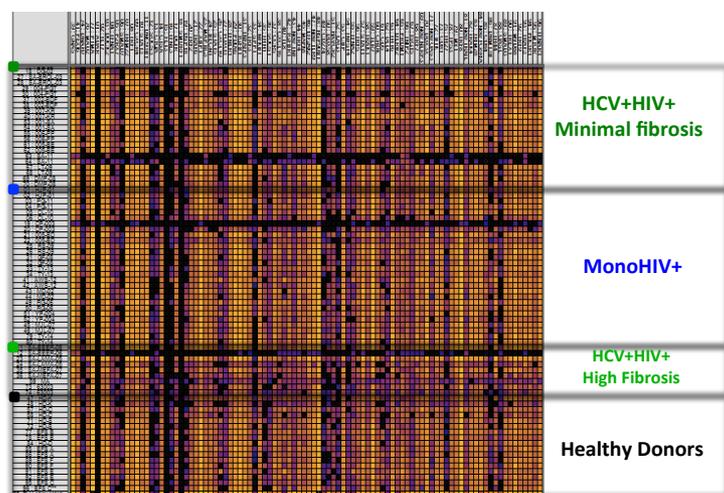
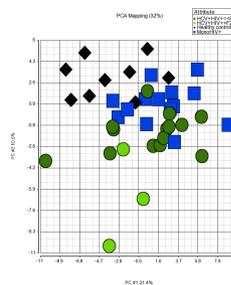
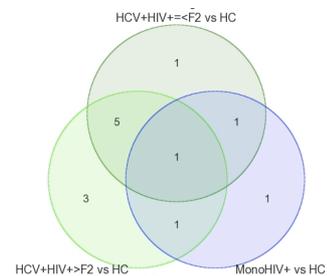
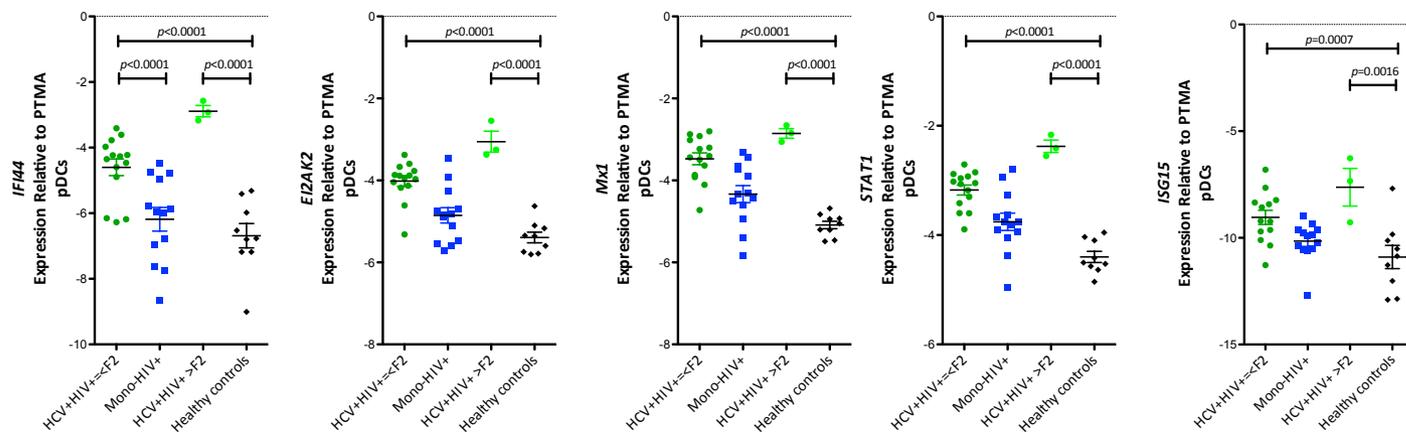
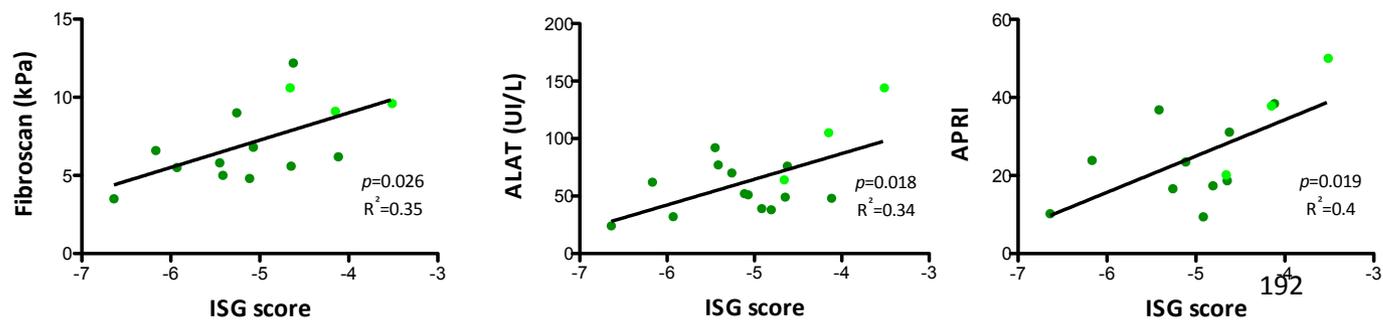


## D



# Figure 3



**Figure 4****A****B****C****D****E**

## Supplementary Figures and Tables

### **Supplementary Figure 1: Comparison of the activation of cDCs and monocytes between HCV-HIV-1 coinfecting individuals under suppressive ART, HIV-1 mono-infected individuals under suppressive ART and healthy controls.**

Fresh whole blood was stained for T-cell costimulatory markers CD40 and CD86 and the hemoglobin scavenger receptor CD163. Expression of markers of activation on cDCs (A), classical CD14<sup>++</sup>CD16<sup>-</sup> (B), non-classical CD14<sup>+</sup>CD16<sup>+</sup> (C) and intermediates monocytes (D) are similar between HCV-HIV-1 coinfecting individuals under suppressive ART and HIV-1 mono-infected individuals under suppressive ART. Comparison between groups was performed by Mann-Whitney t test.

### **Supplementary Figure 2: T cell activation and immunosenescence in HIV-1-HCV co-infected individuals, HIV-1 mono-infected individuals and healthy controls**

Fresh whole blood was stained for the markers of T cell activation CD38 and HLA-DR and for the marker of immunosenescence CD57.

(A) Gating strategy for pDC sorting is shown

(B, C) Representative flow plots from one HIV-1-HCV coinfecting individual, one HIV-1 mono-infected individual and one healthy donors are shown for HLA-DR and CD38 co-expression (B) and CD57 expression (D) on CD8<sup>+</sup> T cells.

### **Supplementary Figure 3: Decreased peripheral pDC numbers in HCV-HIV-1 co-infected patients with advanced fibrosis and association with %CD57<sup>+</sup>CD8<sup>+</sup>**

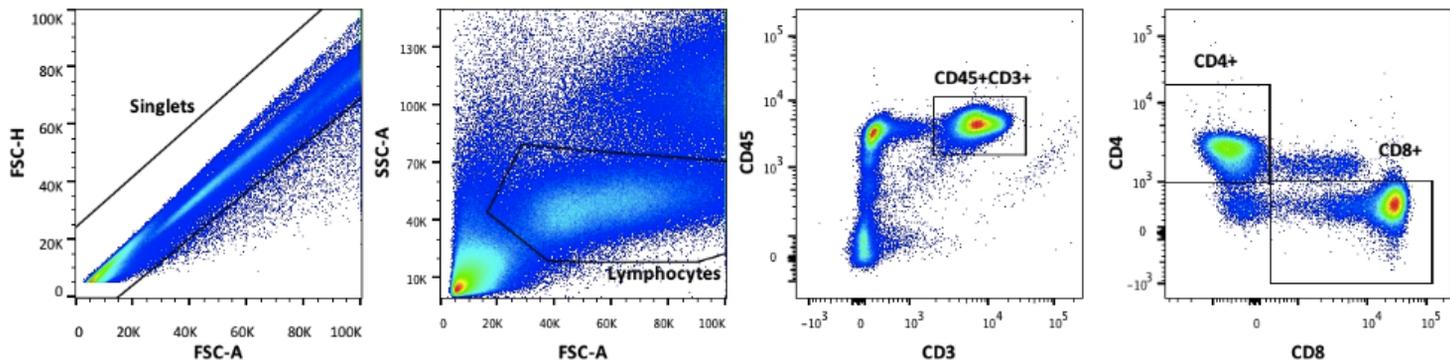
Gating strategy for absolute quantification with the TruCount method (A). Comparison of pDC absolute counts in the peripheral blood between HCV-HIV-1 co-infected individuals with minimal and advanced fibrosis, HIV-1 mono-infected individuals and healthy controls (B). The percentage of CD57<sup>+</sup> CD8<sup>+</sup> T cells was correlated to the absolute pDC counts in the peripheral blood (C). Linear regression was calculated with Spearman rank-based correlation. Comparison between groups was performed by two-tailed Mann-Whitney t test. Horizontal bars show median.

### **Supplementary Figure 4: ISGs expression in PBMCs from HCV-HIV-1 co-infected individuals, HIV-1 mono-infected individuals and healthy controls**

The relative expression of *E12AK2* (A) and *Mx1* (B) in PBMCs is compared between 15 HCV-HIV-1 co-infected, 13 HIV-1 mono-infected individuals, 4 healthy controls and 4 HIV-1-HCV co-infected individuals with severe fibrosis. Horizontal bars show median.

# Supplementary Figure 1

**A**

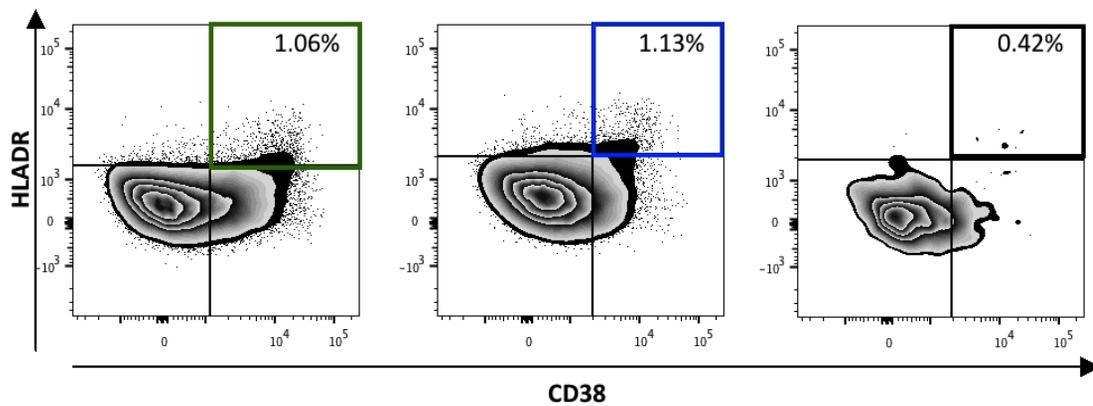


**B**

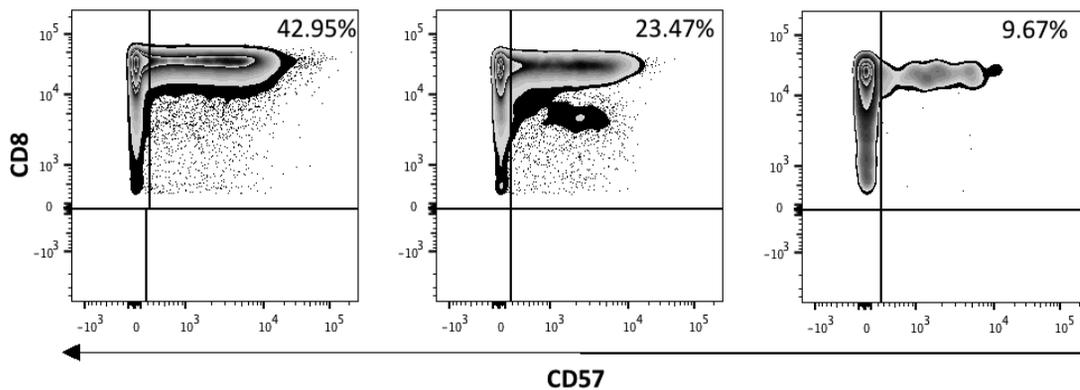
HCV-HIV-1

HIV-1

Healthy control

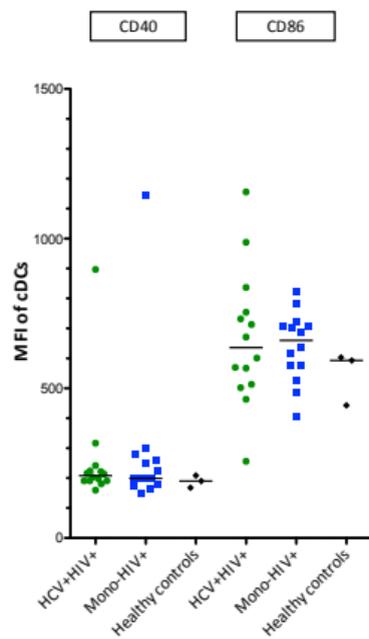


**C**

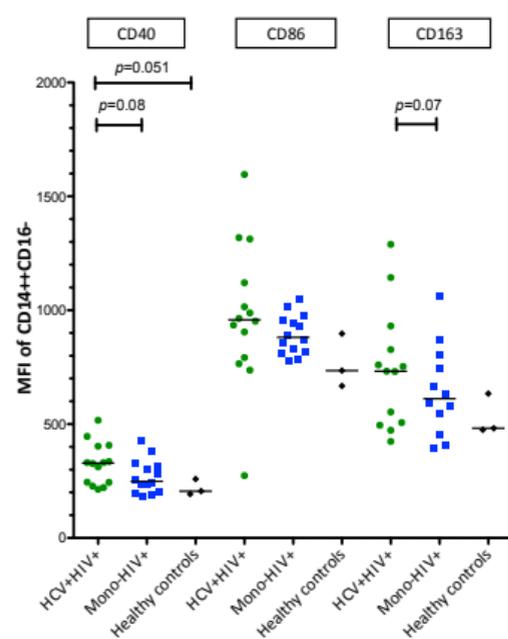


# Supplementary Figure 2

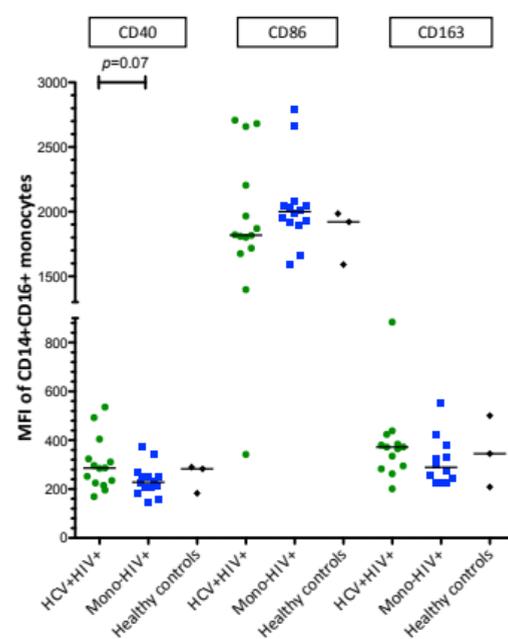
## A



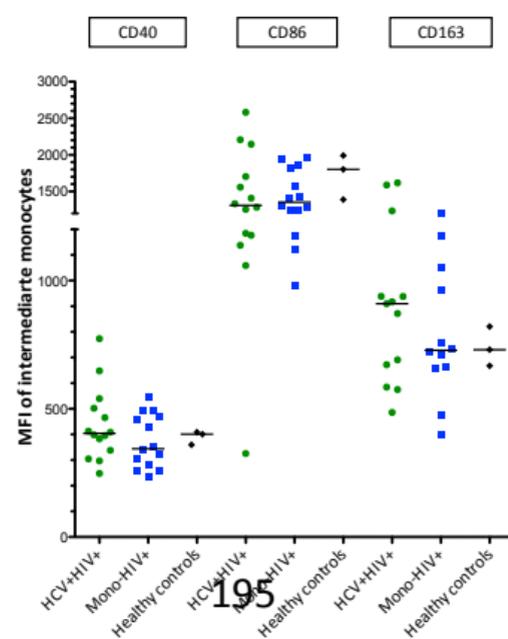
## B



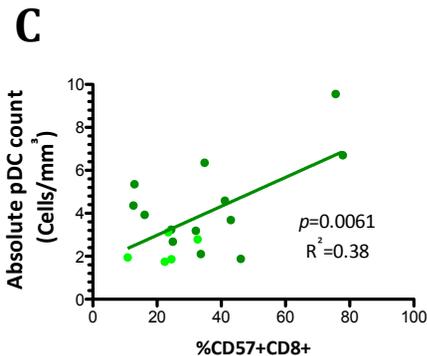
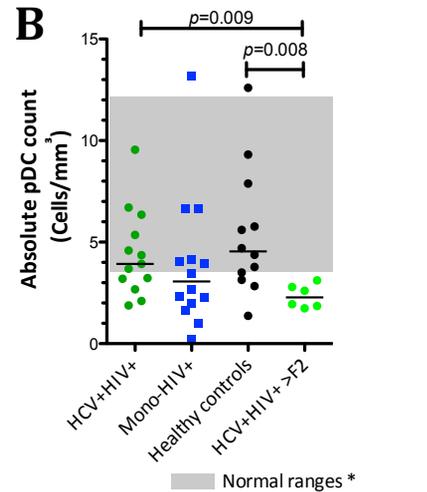
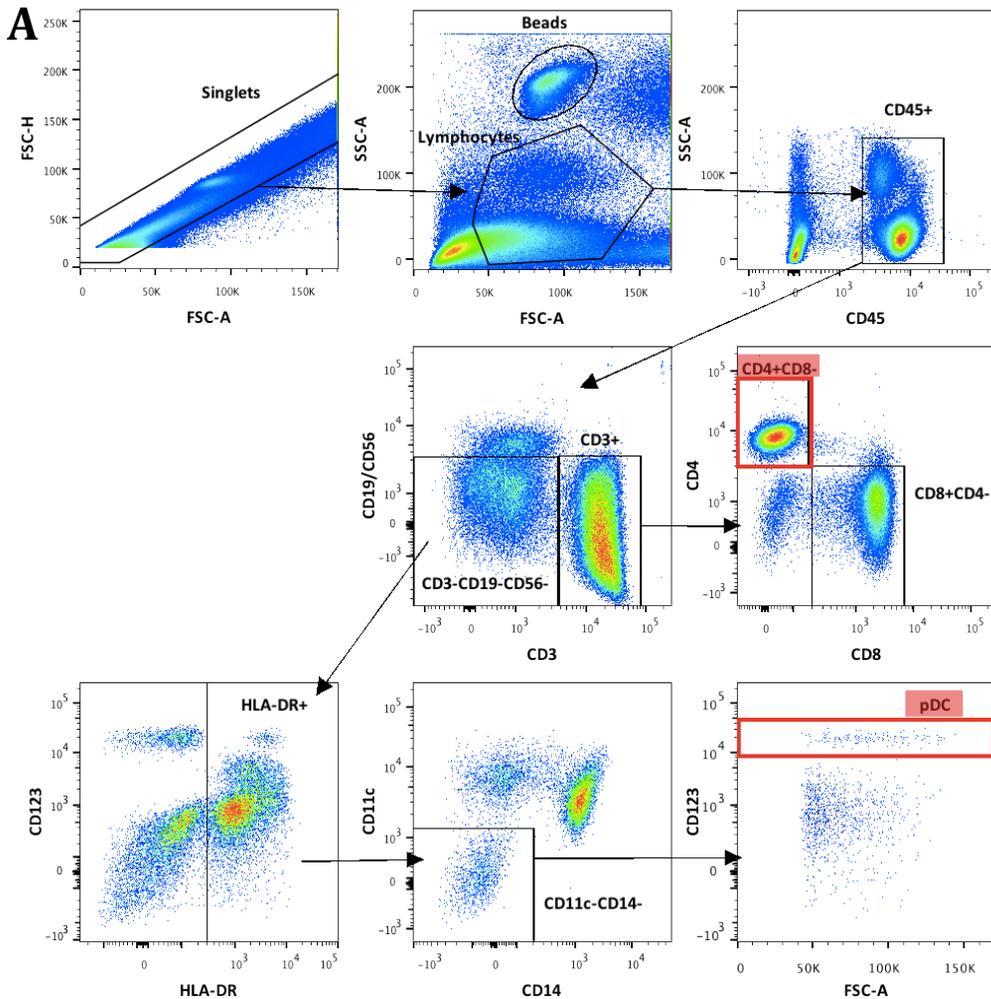
## C



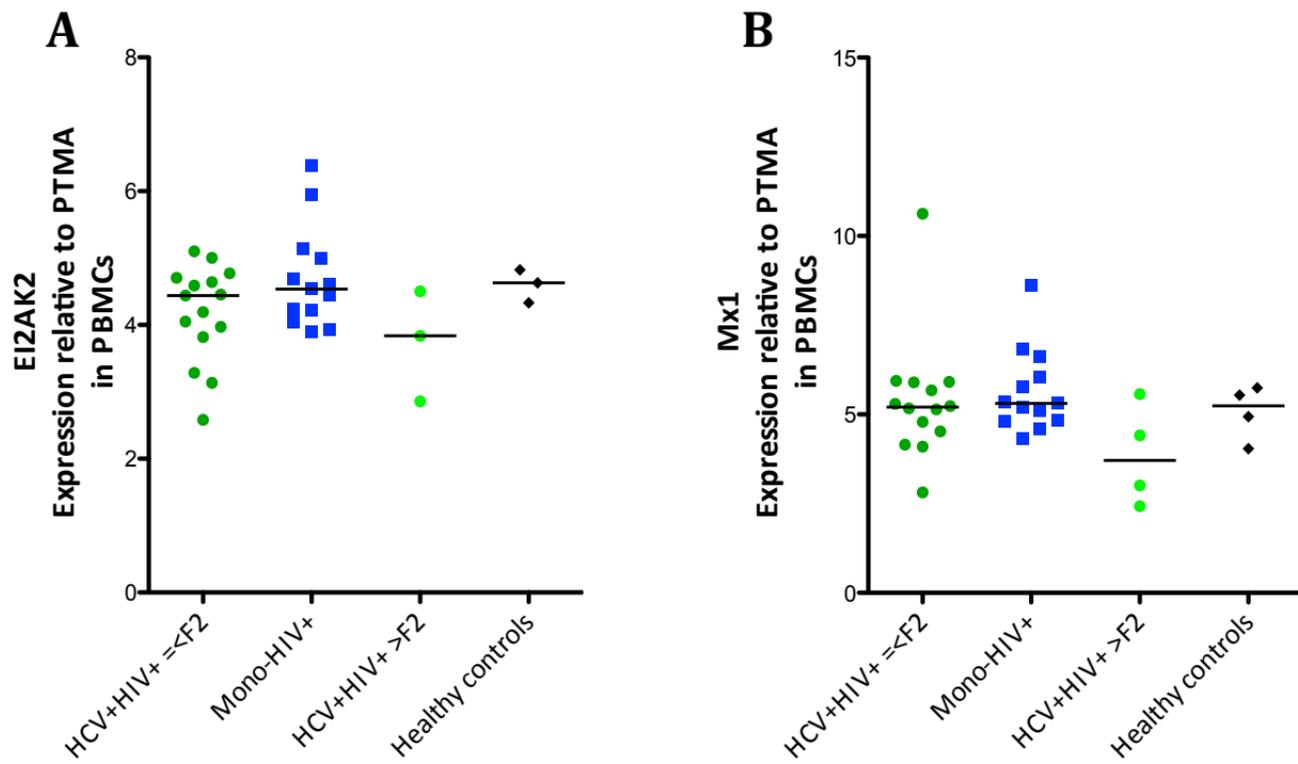
## D



# Supplementary Figure 3



# Supplementary Figure 4



**Supplementary Table 1: Medians of plasma levels of inflammatory markers in HCV-HIV-1 co-infected individuals, HIV-1 mono-infected individuals and healthy donors**

	LLOQ pg/mL	HCV-HIV-1+	Mono-HIV-1+	Healthy controls	p-value HvsHC	p-value MvsHC	p-value HvsM
IFN $\gamma$	0.3	0.3	0.55	0.3	0.29	0.12	0.24
IL-6	1	4.46	1.15	1	0.42	0.27	0.315
IL-10	0.1	1.36	0.3	0.74	0.36	0.31	0.13
sCD40L	3.7	680.2	487.1	402.6	0.053	0.44	0.079
ITAC (CXCL-11)	21.4	149.5	71.19	56.24	0.063	0.76	0.035
TRAIL	8.5	20.92	8.5	8.5	0.063	1.0	0.073
MIP-1 $\beta$	5.2	17.32	11.4	8.05	0.067	0.65	0.028
IP-10 (CXCL-10)	2.5	34.76	9.92	8.57	0.012	0.53	0.0007
IL-8	2.1	2.495	2.1	2.1	0.37	0.63	0.022
TGF- $\beta$	1.7	166	223	126.1	0.065	0.054	0.54
pan-IFN $\alpha$	3	113.9	81.06	62.12	0.003	0.01	0.06

H : HCV-HIV-1 co-infected individuals

M : HIV-1 mono-infected individuals

HC : Healthy controls

Values are in pg/mL

Mann-Whitney t test with correction for multiple tests (Benjamini et Hochberg, 1995)

**Supplementary Table 2 : Characteristics of the 5 HCV-HIV-1 co-infected individuals with moderate to severe fibrosis**

(A) Demographic and HIV-1 related characteristics

Group	HIV-1-HCV-co-infected individuals With advanced fibrosis
	n=5
<b>General characteristics</b>	
Gender	Male
Age (year) (range)	55.3 (44-64.5)
<b>Ethnicity</b>	
Caucasian, number (%)	3 (60%)
African, number (%)	0 (0%)
Maghrebien, number (%)	2 (40%)
West Indian, number (%)	0 (0%)
Unknown, number (%)	0 (0%)
BMI $\geq$ 25 kg/m <sup>2</sup> (%)	1 (16.6%)
<b>History of IDUs</b>	
Active, nombre (%)	0 (0%)
Past, nombre (%)	3 (60%)
No, nombre (%)	2 (40%)
<b>HIV-1 disease characteristics</b>	
HIV-1 Plasma Viral Load (copies/mL)	<20
CD4 absolute median (cells/uL) (range) *	665.8 (388-1044)
CD4 median % (range)*	31.2 (19-40)
CD8 absolute median (cells/uL) (range) *	892.8 (418-1267)
CD8 median % (range)*	39.5 (26-46)
Ratio CD4/CD8 median (range) *	0.86 (0.41-1.54)
<b>Nadir CD4</b>	
<200, number (%), range	4 (80%)
200-350, number (%)	1 (20%)
>350, number (%)	0 (0%)
Estimated duration of HIV-1 infection	

Years (Range)	21.6 (15-31)
> 10 years (%)	5 (100%)
ARV Therapy Years of ART (range)	16.2 (4-21)
Years between diagnostic and HAART (range)	5.4 (0-27)
ART Regimen	
PI-based regimen (%)	0 (0%)
Current use of atazanavir (%)	0 (0%)
Former use of maraviroc (%)	0 (0%)

(B) Patients characteristics relative to HCV infection and fibrosis stage

Patient ID	Estimated duration of HCV infection (Years)	Previous HCV treatment	HCV treatment response	Date of HCV treatment interruption	HCV VL (UI/mL)	HCV genotype	Fibrosca n (kPa)	METAVIR score	ALAT (IU/L)	ASAT (IU/L)
SA-LAMA-05	21	Peg-IFN+Rib		Nov. 2002	613225	1b	9.6	F3	144	90
SA-BEER-06					1374000	1b	9.1	F2	105	62
SA-MEFA-07	15	Peg-IFN+Rib		Sept. 2011	174500	1a	10.6	F3	64	49
MA-15	17	Copegus, Ingivo, Pegasys			663825	1a	21.5	F4	121	106
SA-DE-AL-08*	20		NR	Dec. 2001	5697000	NA	25.7	F4	110	90

NA : Not available, not applicable

NR : Non-responder

\*SA-DE-AL-08 was not used in the transcriptomic study.

**Supplementary Table 3 : Medians of activated and senescent T cell subsets in HCV-HIV-1 co-infected individuals, HIV-1 mono-infected individuals and healthy donors**

	HCV-HIV-1+	Mono-HIV-1+	Healthy controls
Median %CD8+CD38+HLADR+	0.66	0.99	0.31
Median %CD8+ HLADR+	1.02	1.70	0.50
Median %CD8+CD57+	33.9	23.9	0.0028
Median %CD4+CD38+HLADR+	0.92	1.16	1.23
Median %CD4+ HLADR+	2.41	2.94	2.7
Median %CD4+CD57+	3.76	3.42	1.3

**Supplementary Table 4: Differentially expressed genes (DEGs) in pDCs between HIV-1-HCV co-infected individuals with minimal to moderate fibrosis (A), with severe fibrosis (B) and HIV-1 mono-infected individuals (C) compared to healthy controls**

(A) DEGs between HIV-1-HCV co-infected individuals with minimal to moderate fibrosis and healthy controls

Genes	<i>p</i> -value	Fold-change	Description vs healthy controls
STAT1	7.02E-08	2.33557	Up
EIF2AK2	7.17E-07	2.59401	Up
Mx1	1.00E-07	3.05902	Up
IFI44	8.45E-05	4.24427	Up
ISG15	0.00166034	3.62286	Up
PRDM1	0.000645494	-2.72307	Down
IFIH1	0.00150261	2.75186	Up
TNFSF4	0.00370879	-3.62752	Down

(B) DEGs between HIV-1-HCV co-infected individuals with severe fibrosis and healthy controls

Genes	<i>p</i> -value	Fold-change	Description vs healthy controls
STAT1	2.10E-08	4.06583	Up
EIF2AK2	1.42E-07	5.05081	Up
Mx1	9.73E-07	4.7017	Up
IFI44	9.09E-06	13.9041	Up
IFI16	4.77E-05	2.02658	Up
ISG15	0.000413892	9.65363	Up

IFIH1	0.000274598	5.89775	Up
CD274	0.00593387	22.3403	Up
CXCL16	0.0039712	-2.56416	Down
IRF1	0.00618855	2.02185	Up

(C) DEGs between HIV-1 mono-infected individuals and healthy controls

Genes	<i>p</i> -value	Fold-change	Description vs healthy controls
PRDM1	0.000193624	-3.09589	Down
IFIH1	0.00251645	2.63376	Up
CD274	0.00402735	4.79355	Up
IFNA1	0.0036412	-3.82865	Down

**Supplementary Table 5: Correlation between individual ISG levels in pDCs and HCV viral load in pDCs and different measures of fibrosis**

		<i>IFI44</i>	<i>Mx1</i>	<i>ISG15</i>	<i>EIF2AK2</i>	<i>STAT1</i>
<b>ALAT</b>	<i>p</i>	0.0403	0.0743	0.0571	0.0014	0.0094
	R <sup>2</sup>	0.2515	0.197	0.2348	0.5032	0.3712
<b>Fibroscan</b>	<i>p</i>	0.0308	0.0251	0.1236	0.009	0.0012
	R <sup>2</sup>	0.3328	0.3527	0.1861	0.4465	0.5969
<b>APRI</b>	<i>p</i>	0.0524	0.0384	0.031	0.0055	0.1826
	R <sup>2</sup>	0.2786	0.3106	0.3572	0.4867	0.1429

# Supplementary Table 6

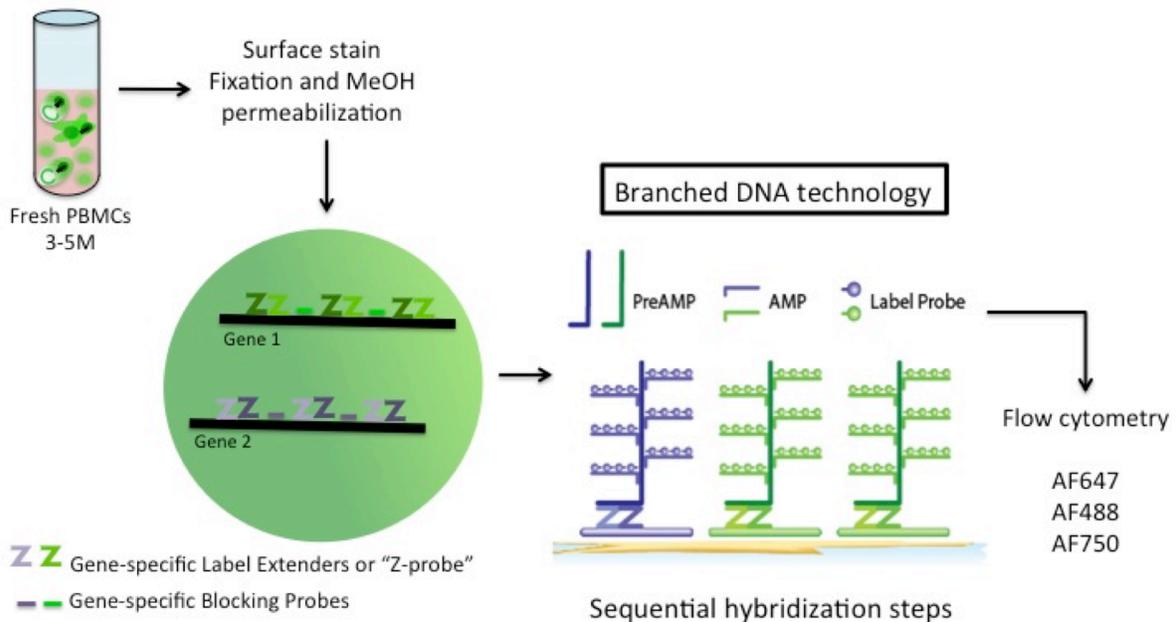
Target	Forward Primer	Reverse Primer	Design RefSeq
A20	GAAGCTTGTGGCGCTGAAAA	CCTGAACGCCCCACATGTA	NM_006290.2
ADAR	GCACCTGTGACCCACTCTCC	CAGATGCCCTTGGCTGAAAA	NM_001025107.2
Beclin-1	GGTTGAGAAAGGGCGAGACAC	ACTGCCTCTGTGCTTCAA	NM_003766.3
BTX	CATGGTGAGAGACGAGATAA	GGAGTCTTCTGCTCAGTGACA	NM_000061.2
C1QA	AGCATCCAGTTGGAGTTGACA	ACAGAGCACCCAGCCATCC	NM_015991.2
CBP	AACCAGCACAGCCTCAAAC	TCCTCTGCTGGAATGTTCA	NM_001079846.1
CCRS	TGAGACATCCGTTCCCTACA	TGGCAGGGTCCGATGTATA	NM_001100168.1
CD274	GGAGATTAGATCCTGAGAAAAACCA	TGGAGGATGTGCCAGAGGTA	NM_014143.3
CD5	CCCTTGCTCAAGAAGCTA	TCCCGTTGGCCAATCC	NM_014207.3
CD81	GGCAGCAACATCATCAGCAA	AGCAATGCCGATGAGGTACA	NM_004356.3
CD84	TCCAAGGAAGCCAGTGAAC	GCTTGAAGTCCAGGAGGTTTA	NM_003874.3
CTSA	GATGAGTGGTTTGTGGATTCCC	GCTGTCCCGCTACTTCACTAA	NM_001167594.1
CXCL10	GCTGTACTGCATCAGCATTAA	CTGGATTACAGACATCTCTCTCAC	NM_001565.2
CXCL16	GGAAACACCTGAGAGCTTACCA	CCACACACCTCCAGGAA	NM_022059.2
CXCR4	ATCTTCTGCCACCATCTAC	CCCATGACCAGGATGACCAA	NM_003467.2
DCIR	GGCAAGACAGTGAAGAAGGAC	GAGAGCCCCACAAAATAGCA	NM_194447.2
DDX3X	AGCTTCTCAGTGATCGTGGAA	TGCCAATGCCATCGTAATCAC	NM_001193417.1
DDX58	GACTGGACGTGGCAAAACA	CTCCTACTGGCTTGAATGCA	NM_014314.3
DL14	CTCAGCGAGTGTGACAGCAA	GACACAGCAGTGGTATGCC	NM_019074.3
DNAH7	GGTAATCCCTAGGCTGGTTA	GTGCAAATGAAGGGCTCCAA	NM_018897.2
DNMT3B	GTGAAGCACGAGGGGAATATCA	TTCCGCCAATCACCAAGTCA	NM_006892.3
DOCK2	ACTTTGGGAATGGGAAGCA	ATGGACTGCACCTGGAGAAAA	NM_004946.2
E2F2K2	TGCCATAATCAGGACCTCCA	TCACCTTCTGGAATTTCTCTCC	NM_002759.2
FCeR1a New	ataaaaGctccggtgagaa	tcaccaccaaatgggata	NM_002001.2
FLT3	AAACGGCCATCTTCCCTTAA	ACGGCCATCCACATTCTGATA	NM_004119.2
FOXO3	CGCTCTCTCGCTCGAA	TTTTCAGGGGCGCCAGTA	NM_001455.3
GNAS	CAACGAGTACCAGCTGATTGAC	GATCGCTCCGACATAGTCA	NM_001077490.1
gp96	GTTCCCGGAGACTCTTCA	CATGTCCAGGTTTTACGAACAA	NM_003299.1
HAVCR2	GGATCAAATCCAGGCATAA	CTTGAAAGGTGCAGTGAA	NM_032782.3
IFI16	GTGAATGGGGTGTGGAGGTAC	CACCACTTCATCTTCCCTGTA	NM_005531.2
IFI27	TTGTGGTACTCTGCAGTCA	CCCAGGATGAATTTGGTCAA	NM_005532.3
IFI44	GGCTTTGGTGGGCACTAATA	TGCCATTTTCCCGTCTCTA	NM_006417.4
IFNA1	TGACTCATACACAGGTAC	CAGGGGTGAGAGTCTTTGAA	NM_024013.2
IFNA2	TCTCTGCTTGAAGGACAGAC	GGAACTGGTGGCCAACTCC	NM_000605.3
IFNAR1	AGTGACGCTGTATGTGAGAAAA	ACGGGAGAGCAAAATAGTCA	NM_000629.2
IFNL3	GTCCACATAGCCAGTCAA	CGGCACTTGCAGTCTTCA	NM_172139.2
IFNLR1	CCTGGGTGGAGTCCGAATAC	CTGGGTGAGCACCAAGGAC	NM_173065.1
IKKalpha	GAGAACGATGTCATGAA	GGAAACATCACAGGCTTTACA	NM_001278.3
IL8	ACACTGCGCCAACACAGAAA	CAGTTTTCTTGGGGTCCAGAC	NM_000584.2
IL7	acacaacgtctctccgagt	tgtCTcgcgacaggatgt	NM_012276.3
IRAK1	AGTGAAGCAGAGTCTCTGAC	TGAGCACAGTAGCCAGCAAA	NM_001025243.1
IRAK4	GCCTGGAGAAATAACACCCAAA	TCATCCACAGCTGGAAGTCC	NM_001145258.1
IRF1	AACAAGGATGCTGCTTTTCTCC	TGGGATCTGGCTCTTTTCC	NM_002198.2
IRF2	TGGCTGGAGGAGCAGATAAA	ATGCATCCAGGGGATCTGAA	NM_002199.3
IRF3	CCAGCCAGACACTCTCC	TGGGGCCACACCATGTTA	NM_001197122.1
IRF4	CTACAACCCGAGGAGGAC	GTCTGATGCTTCTCGGAAC	NM_002460.3
IRF5	AGATCTACGAGTCTGCTCAA	CCTCTCTGCACCAAAAGAGTA	NM_002200.3
IRF7	GGCAGAGCCGTACTGTCA	ACCGTGGCCCTTGTGA	NM_004031.2
IRF8	TGGTCCAGTCTTCGACAC	CGGCCCTGGCTTATAGAA	NM_002163.2
IRF9	TGCTCCAGGACTCCCTCAA	TGCTGCTCCAAATGTCTGAA	NM_006084.4
ISG15	CTGAGAGGCGAGGAACTCA	GCTCAGGGACACTGGAA	NM_005101.3
JNK1	TCTCAAACCCCTACATCA	CCCTTTCATTAACCTGTTGCTA	NM_002750.2
LAG3	TGGAGCCTTTGGCTTTCAC	GAGGGTGAATCCCTTGTCTCA	NM_002286.5
LC3B	AGTACAGCGGAGAAACACA	CTTAAAGCCGAGGAGCAGAA	NM_022818.4
LILRB2	TCAACTCCAGCCCTACCT	GGGCTGGAACCCATGGA	NM_005874.3
MAU2	CCAACCTGGCGAGTGTATA	TCCGGTGTATCTCTCCA	NM_015329.3
MDA-5-IFIH1	GGTCTGGGGCATGGAGAATAA	CTGACACTCTCTGCCAAAC	NM_022168.2
mTOR	CCAAACCCAGGTGTGATCAA	TCCTATTTCAGGCCACTA	NM_004958.3
MX1	ATGCTACTGTGGCCCAAGAAA	GGCGCACCTTCTCTCTATA	NM_001144925.1
MyD88	CTGCAGAGCAAGGAATGTGAC	TGCTGGGAACTCTTTCTCA	NM_002468.4
NF-KB1	TGTGAACACCCCTCTAGATA	AAACTCTGGCTCATATGGTTTCC	NM_003998.3
NLRP3 New	tttatgagaaagcaaaagagatg	gaaCCcaactcggctcat	NM_001079821.2
OAS3 New	GGATGGATGTGACCTgggtg	cctgaCCcaggacattgaag	NM_006187.2
OASL	ACCAATTGCTCGCTACACA	GGCCTTGATCAGGCTCACATA	NM_003733.3
OPN (SPP1)	CCAGCAACCGAAGTTTTCAC	TGATGTCCTGCTGTAGCA	NM_000582.2
P2RX7	CTTCGGGATCCGTTTGGACA	TTGAGCCGATGTACACAACC	NM_002562.5
PDI46	AGGTTCTGGCCTCCGATA	ACCGTCATAATCCACAGGAGAC	NM_005742.2
PEX16	CGATTCCGACGAGCTGTCA	AAGCTCTCCGAGGATCCC	NM_057174.2
P13KR1	CGAGTGGTTGGGCAATGAAAA	GTTGCTGCTTCCAACTTCCA	NM_181504.2
PRDM1	CTGGTACACACGGGAGAAAA	TTGAGATTGCTGCTGCTCA	NM_001198.3
PTMA	GGAGCAGGAGGCTGACA	AGCTTCTCATCTTCTATCCTCA	NM_002823.4
SMAD3	ACCAGTACCACAGATGAA	GGACATCGGATCGGGGATA	NM_005902.3
SMAD7	GTGCTTCTCCGCTGAAA	CGATCCCCAGGCTCCAGAA	NM_005904.3
SMURF1	AGCTTTTGACCAAGGAAAC	GTTGACTTCCAGTCTTCAA	NM_020429.1
SOC51	CATCCGCTGCACTTTCA	GCTCGAAGAGCGAGTCAAA	NM_003745.1
SOC53	GCTCAGCCCAAGGAC	GAAACTTGCTGGGTGAC	NM_003955.3
STAT1	TACCTGGCACAGTGGTTAGAA	TGAAAACGGATGGTGGCAAA	NM_007315.3
STAT3	GGAAATAATGGTGAAGGTGCTGAA	CCGAGGTCAACTCCATGTCAAA	NM_003150.3
STAT5	CCAGGCTCCCTATAACATGTA	ATGGTCTCATCCAGGTCGAA	NM_003152.3
TGFB1	CGTCTGCTGAGGCTCAAGTTA	TCGCCAGGAATTGCTGTA	NM_000660.4
TGFBR1	GAAATTGCTCGACGATGTTCC	ACTGATGGTCCAGAGGTACA	NM_004612.2
TLR7	TCTTCAACAGACCTCTACATTCC	AGCCCCAAGGATTTGGAAA	NM_016562.3
TLR9 New	ccccagcatGggttctg	GAGAGACgagggtgacg	NM_017442.2
TNF New	tgtGCaacccctcaagctg	attggccaggaggcatt	NM_000594.2
TNFSF4	CACATCGGTATCCTGAAATCA	TGATTTCTACTCTTTTGGGAA	NM_003326.3
TNIP2	CGGGTGCAGATGCTGGAA	CCTACTTTGAGCCGTTCC	NM_024309.3
TP53	TGAATGAGGCTTGGAACTCA	TCAGGCCCTTCTGCTTCAA	NM_000546.4
TRAF3	AGAGGTTGTGCAGAGCAGTTA	CGCACACATGGAAAGTTCTCA	NM_145725.1
TRAF6	TGGCAAAATGTCATCTGGAATAC	GCTGTAGGGCAGTCTAGATCA	NM_004620.2
TRAIL New	tcagtcatgatgatgagctg	gatcacagcaggtctgt	NM_003810.3
TREX1	ACCATCTGCTGCACAAACA	TCTCTCAAAGGCTGGACTA	NM_033629.3
TRIM22	CTCCCTGATTCAAGACTCC	GCAGTCCACTCTTCTCTA	NM_006074.4
TRIM28/KAP1	AGCGGGTGAAGTACACAA	AGACAGTACTTCCACTCCC	NM_005762.2
UNC93B1	CTGAACACACGCTGTACAA	CGCAGAACCCTTGTGAA	NM_030930.2
USP18	TGAAATGGACTTCCAGGATA	GCAGCAGAAGCATCTGGAAA	NM_017414.3
XBP1	CATGGATTCTGGCGTATTGAC	TGGTCCAAGTTGTCCAGAA	NM_005080.3



## Methods



## Validation and application of the QuantiGene® Flow RNA assay to the study of pDC IFN $\alpha$ pathway



**Figure M1: Principle and workflow of the QuantiGene® FlowRNA Assay**

Target-specific oligonucleotide pairs hybridize to target mRNAs within fixed and methanol-permeabilized cells. Typically 20-40 "Z-probes" hybridize on a single RNA molecule and serve as anchor for signal amplification achieved thanks to the branched DNA technology. DNA branches are sequentially hybridized during pre-amplification and amplification, followed by staining with labeled probes. Signal can therefore be amplified up to 8,000 fold. Simultaneous detection of up to three RNA targets in a single cell is rendered possible by the use of independent but compatible signal amplification systems associated with the fluorochromes Alexa 488, 647 and 750.

The QuantiGene® Flow RNA assay is based on fluorescent in situ hybridization (FISH) and allows for simultaneous detection of up to three RNA transcripts in a single cell using a standard flow cytometer. The principle and workflow of QuantiGene® Flow RNA protocol are outlined in Figure M1. The assay has several advantages including (1) simple workflow and non-requirement for cell sorting, (2) unrestricted target availability as detection is sequence- and not antibody-based, (3) single-cell level of detection. Anecdotally, we were the first official FlowRNA customer in North America. Our goal was to implement the novel FlowRNA technique to the study of pDCs IFN $\alpha$  responses. The FlowRNA protocol is incompatible with intracellular staining (a novel version is compatible though). We decided to combine the FlowRNA to the Miltenyi IFN $\alpha$  secretion assay to simultaneously detect IFN $\alpha$  mRNA induction and protein secretion by pDCs upon TLR7 stimulation. We

are presenting here our protocol validation and how it can be easily utilized to investigate to an unprecedented level of detail pDC IFN $\alpha$  response.

### **Establishment of the QuantiGene® FlowRNA assay protocol to simultaneously measure IFN $\alpha$ secretion and *IFNA2* mRNA**

First, we identified surface staining conditions withstanding the procedures (including methanol permeabilization) and obtained appropriate definition of cell populations as depicted on Figure M2A. We then checked the specificity of the detection of *IFNA2* mRNA and secreted IFN $\alpha$ . The detection of secreted IFN $\alpha$  is based on the trapping of IFN $\alpha$  by a primary antibody (capture antibody) coated on the cell surface, followed by detection with a secondary fluorescent-labeled antibody (detection antibody). We use detection antibody in the absence of capture antibody and PE-labeled isotype instead of the detection antibody (in the presence of capture antibody) to fully account for the specificity of detection of secreted IFN $\alpha$ . We did not observe any unspecific detection (Figure M2B). Specificity of mRNA detection was controlled using a probe recognizing bacterial RNA (Figure M2B). Some low levels of *IFNA2* mRNA were detected at baseline in pDCs, in accordance with endogenous IFN $\alpha$  expression in pDCs. *IFNA2* was upregulated upon TLR7 stimulation in pDCs. As expected, no *IFNA2* mRNA upregulation or IFN $\alpha$  secretion were seen in T cells upon 2 hours of TLR7 stimulation (Figure M2B). Importantly, the FlowRNA assay requires the use of harsh buffers, which can affect the capture of secreted IFN $\alpha$ . Hence, we assessed the robustness of the detection of IFN $\alpha$  secretion. While a small reduction in the percentage of IFN $\alpha$ -secreting pDCs using the FlowRNA protocol compared to regular surface stain was observed, there was no significant difference between the two protocols (Figure M2C). Subsequently, reproducibility was evaluated in four samples, each run in triplicates. A very good reproducibility between triplicates was obtained (Figure M2D). Finally, detection of *IFNA2* mRNA by FlowRNA was compared to its detection by qRT-PCR. CL097-stimulated PBMCs were split in half. IFN $\alpha$ -secreting and non-secreting pDCs were sorted from the first half and *IFNA2* mRNA levels were determined by qRT-PCR in both fractions. The other half was subjected to the FlowRNA protocol and the MFI of *IFNA2* mRNA was determined in IFN $\alpha$ -secreting and non-secreting pDCs. Both methods show a higher *IFNA2* mRNA levels in IFN $\alpha$ -secreting than non-secreting pDCs and similar patterns of distribution for the three individuals tested. Altogether, these data show that the FlowRNA method enables to sensitively and robustly detect *IFNA2* mRNA and secreted IFN $\alpha$  in the same pDCs.

### **Utilization of FlowRNA method to uncover functional heterogeneity of pDC**

We first asked whether the kinetics of *IFNA2* mRNA induction and IFN $\alpha$  secretion in pDCs were similar. We found a positive correlation between the percentages of *IFNA2*-expressing and IFN $\alpha$ -secreting pDCs (Figure M3A,  $p=0.038$ ,  $R^2=0.62$ ), suggesting that IFN $\alpha$  protein production is not delayed compared to *IFNA2* mRNA induction. One major advantage of the FlowRNA technique is the single-cell level of detection. By plotting *IFNA2*

mRNA against secreted IFN $\alpha$  in pDCs, we confirmed that *IFNA2*<sup>+</sup> pDCs were all secreting IFN $\alpha$  (Figure M3B). An *IFNA2*IFN $\alpha$ <sup>+</sup> pDC population, more obvious in donor 1 (Figure M3B), was observed. Given that *IFNA2* represents only one of the thirteen IFN $\alpha$  subtypes, it is tempting to speculate that another subtype is induced in this population. This preliminary result suggests that the pDC population may be heterogeneous in regard of the *IFNA* subtype induced. Further work is needed to confirm this hypothesis.

We then asked whether pDCs are heterogeneous regarding the expression of two TLR7-induced cytokines, *IFNA2* and *TNFA*. Simultaneous detection of *IFNA2* and *TNFA* mRNA in pDCs revealed that TLR7-stimulated pDCs segregate primarily into *TNFA*<sup>-</sup>*IFNA2*<sup>-</sup>, *TNFA*<sup>+</sup>*IFNA2*<sup>-</sup> and *TNFA*<sup>+</sup>*IFNA2*<sup>+</sup> cells, with a significantly lower percentage of *TNFA*<sup>-</sup>*IFNA2*<sup>+</sup>pDCs (Figure M3C). This result suggests that the pattern of induction of *TNFA* and *IFNA2* differs among pDCs. The pattern of expression observed may be due to faster kinetics of induction of *TNFA* compared to *IFNA2* or to a higher threshold determining *IFNA2* induction. Nevertheless, the biological relevance of such setting is unclear, as either a lower threshold or a faster induction of pro-inflammatory *TNFA* seem less advantageous as promoting pro-inflammatory rather than antiviral responses. But TNF $\alpha$  protein production may not correlate to *TNFA* mRNA levels. Also, the different patterns may relate to pDCs subsets. Furthermore, the MFI of cytokine mRNA in bulk pDCs population gives the average expression in this population and can therefore be seen as a surrogate measure for values obtained by qRT-PCR, which also provide an average value. Two donors may have similar average expression of TNF but very different population distributions (Figure M3C). Altogether, these data demonstrate the promising potential of this single-cell level detection technique to uncover functional heterogeneity of pDCs.

### **Simultaneous measure of IRF5 mRNA expression in multiple PBMCs subsets by FlowRNA**

We then assessed the high-throughput potential of the technique. We simultaneously profile IRF5 and ESR1 mRNA expression in five PBMC subsets: T cells, B cells, mDCs, monocytes and pDCs. We observed a trend towards higher IRF5 mRNA levels in pDCs from females than in males. Except maybe in B cells, we did not observe any differences in other PBMC subsets. These experiments show the high-throughput capability of the FlowRNA technique.

### **Conclusion**

The protocol established here can yield to a valuable level of information on pDC IFN $\alpha$  pathway levels. We have highlighted the relevance of single-cell analysis for the study of pDC IFN $\alpha$  responses and the high-throughput of the FlowRNA technique. Besides, the protocol does not require to block cytokine secretion to measure either mRNA or protein levels. Because of the short stimulation duration used here, we trapped all the secreted IFN $\alpha$ . Nevertheless, for longer stimulation, it is possible and even recommended to only capture the secreted IFN $\alpha$  in

the last 45 minutes of the stimulation, hence allowing potential cell-cell interactions important for autocrine and paracrine-dependent modulation of activation signals. In conclusion, the protocol established here and more generally the FlowRNA assay show promising potential to better characterize pDCs functional heterogeneity.

#### *Optimized QuantiGene® Flow RNA protocol*

Five million PBMCs were pelleted and surface stained on ice for 30 min. Cells were subjected to the QuantiGene FlowRNA assay (eBiosciences, San Diego, CA) as per manufacturer's instructions with variable type 1 (Alexa Fluor 647) and type 4 (Alexa Fluor 488) probes and type 6 (Alexa Fluor 750)-*B2M* probe used as internal assay control (probes are all from eBiosciences). The bacterial *DapB* probes were used as control for unspecificity. In order to gain sensitivity, target incubation time was increased from 2 hours to 3 hours. Similarly, pre-amplification and amplification incubation times were increased from 1.5 hours to 2 hours. Samples were acquired on the BD Biosciences Fortessa within 2 hours of staining. The mean fluorescent intensity (MFI) of the probes was determined by subsequent analysis using FlowJo software. For experiments combining the IFN $\alpha$  secretion assay (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), the capture antibody was mixed to the cells before stimulation as per manufacturer's instructions and cells were stained with the PE-labeled detection antibody at the end of the FlowRNA protocol in 100 $\mu$ L of storage buffer.

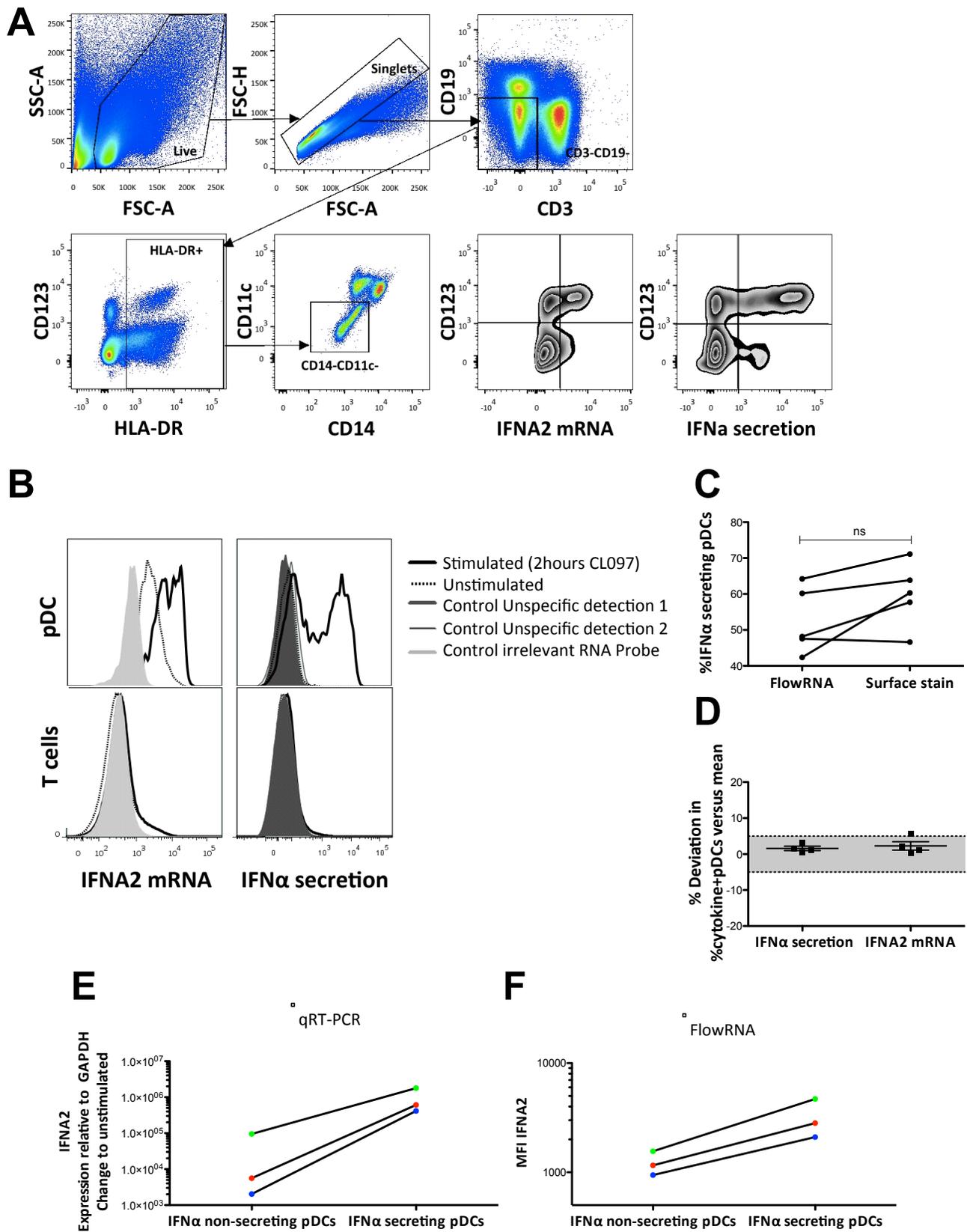


Figure M2: Validation of the QuantiGene® FlowRNA Assay to the study of pDC IFN $\alpha$  pathway

Freshly isolated PBMCs were stimulated for 2 hours with the TLR7 synthetic agonist CL097. Cells were stained for surface markers and IFN $\alpha$  secretion. Samples were subjected to the QuantiGene<sup>®</sup> FlowRNA optimized protocol with type 1 (AF647) IFNA2 probe, type 4 (AF488) TNFA probe and type 6 (AF750) B2M probe.

(A) Gating strategy is shown.

(B) Flow cytometry histogram overlays show the mean intensities of the *IFNA2* mRNA (left) and secreted IFN $\alpha$  (right) in pDCs (top) and T cells (bottom). Control unspecific detection 1 refers to the use of detection antibody in the absence of capture antibody. Control unspecific detection 2 replaces the detection antibody with the PE-labeled isotype (in the presence of capture antibody).

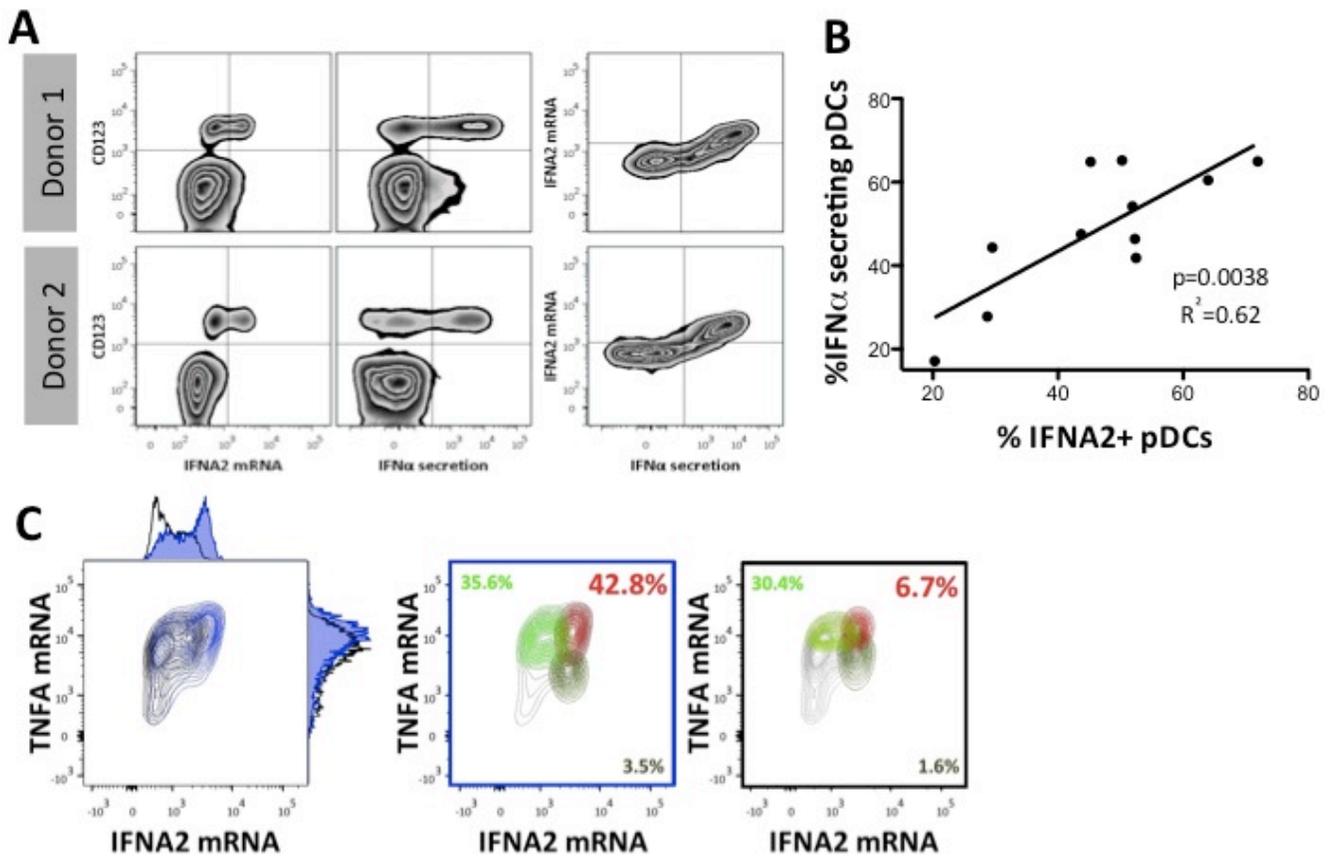
(C) The percentage of IFN $\alpha$ -secreting pDCs is compared between FlowRNA protocol and regular surface staining protocol.

(D) Samples were run in triplicates and the mean of the percentage of IFNA2+ and IFN $\alpha$ -secreting pDCs was calculated. The percentage of deviation in comparison to the mean is plotted. The grey area represents a deviation of  $\pm 5\%$  compared to the mean.

(E,F) Freshly isolated PBMCs from 3 donors (blue, red, green) were stimulated for 2 hours with the TLR7 synthetic agonist CL097 and split in half.

(E) IFN $\alpha$ -secreting and non-secreting pDCs were sorted from half the cells. IFNA2 relative expression was determined by qRT-PCR in both fractions.

(F) The other half was subjected to the FlowRNA protocol. Mean fluorescence intensity (MFI) of IFNA2 mRNA was determined in IFN $\alpha$ -secreting and non-secreting pDCs.



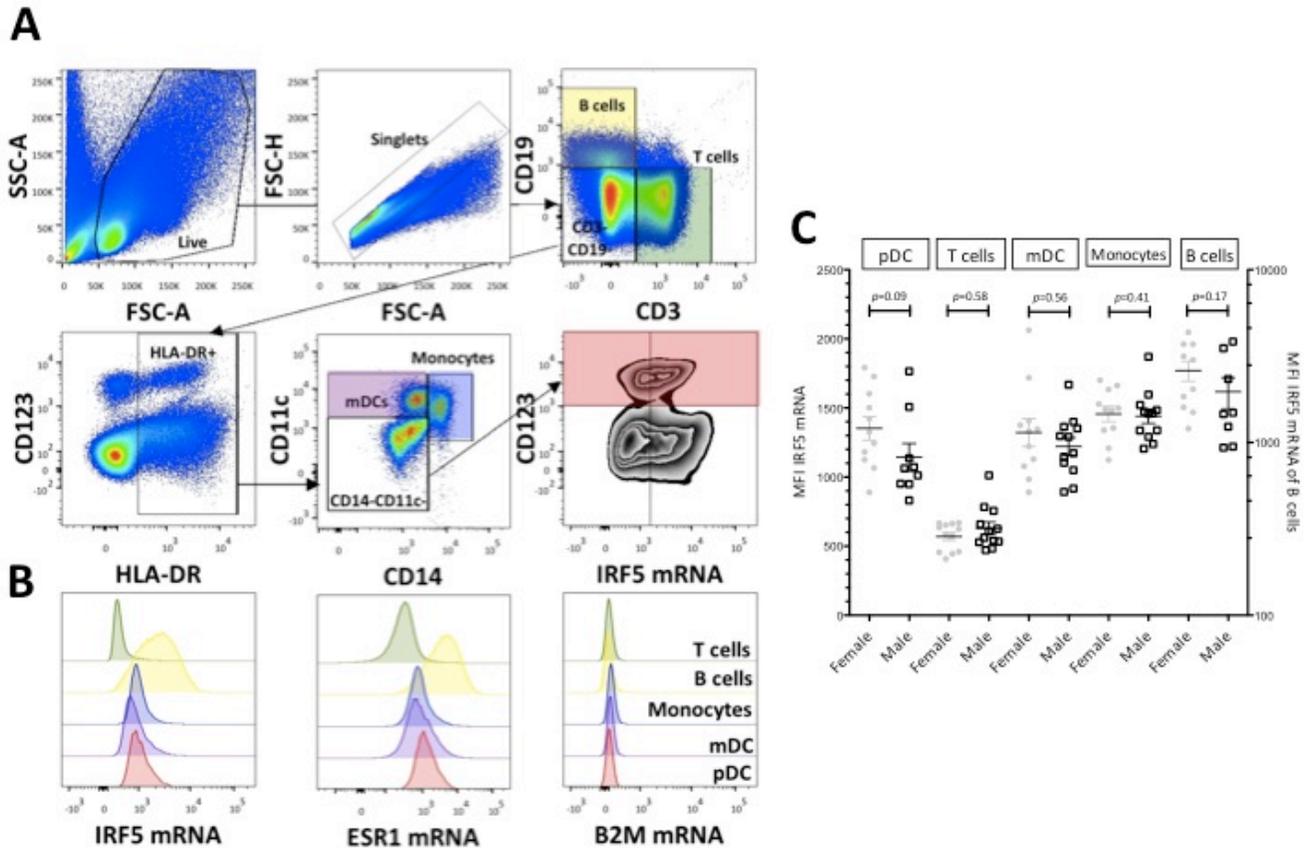
**Figure M3: Comprehensive evaluation of TLR7-mediated cytokine induction in pDCs from single-cell analysis**

Freshly isolated PBMCs were stimulated for 2 hours with the TLR7 synthetic agonist CL097. Cells were stained for surface markers and IFN $\alpha$  secretion. Samples were subjected to the QuantiGene<sup>®</sup> FlowRNA optimized protocol with type 1 (AF647) IFNA2 probe, type 4 (AF488) TNFA probe and type 6 (AF750) B2M probe.

(A) Representative flow plots of *IFNA2* mRNA induction and IFN $\alpha$  secretion by pDCs from two healthy donors

(B) Correlation between the percentage of pDCs secreting IFN $\alpha$  and positive for *IFNA2* mRNA. Linear regression was determined by Spearman rank correlation.

(C) Overlay of the expression of TNFA and IFNA2 mRNA in pDCs from donor 1 (blue) and donor 2 (black). Histograms represent mean fluorescent intensity. Population distribution and percentages of TNFA<sup>+</sup>IFNA2<sup>-</sup>pDCs (light green), TNFA<sup>+</sup>IFNA2<sup>+</sup>pDCs (red) and TNFA<sup>-</sup>IFNA2<sup>+</sup>pDCs (dark green) are shown for donor 1 (middle) and donor 2 (right).



**Figure M4: Utilization of the QuantiGene® FlowRNA Assay to quantify IRF5 mRNA levels simultaneously in multiple PBMCs subsets**

Freshly isolated PBMCs were stained for surface markers and subjected to the QuantiGene® FlowRNA optimized protocol. Samples were stored for less than a week at  $-80^{\circ}\text{C}$  in MeOH. Samples were then stained with type 1 (AF647) ESR1 probe, type 4 (AF488) ultrasensitive IRF5 probe and type 6 (AF750) B2M probe. Samples were run in duplicates. The MFI of *IRF5*, *Esr1* and *B2M* probes were determined by subsequent analysis using FlowJo software. Values were excluded if the duplicates exhibit more than 20% difference. Comparison between sex was performed by Mann-Whitney t test.

## **Discussion, Presentation of supplementary data and Perspectives**



My thesis project falls within the global goal of understanding the mechanisms resulting in deleterious immune activation and inflammation in chronic HIV-1 infection in the post-ART era, with particular focus on the type I IFN pathway. Therapeutic strategies aiming at blocking abnormal pDC IFN $\alpha$  production may limit HIV-1 immunopathogenesis. IFN $\alpha$  affects immune responses in multiple ways but the molecular mechanisms associated to a given immunomodulatory effect are incompletely understood. During my thesis, I focused on dissecting the mechanisms underlying elevated IFN $\alpha$  production by pDCs, and in particular the pathways leading to differences in IFN $\alpha$  production between females and males.

## **I. IRF5 as a mediator of increased pDCs IFN $\alpha$ response in females**

### **1. Results obtained**

Sex differences in pDCs TLR7-mediated IFN $\alpha$  production have previously been described by our group and others<sup>187,259,289</sup> and may involve regulation by estrogen signaling<sup>259</sup>. Nevertheless, the precise mechanisms underlying these differences remained incompletely understood. We demonstrated that pDCs derived from healthy females exhibit higher basal levels of IRF5 than pDCs derived from healthy males and that higher levels of IRF5, including experimental overexpression, are directly associated with higher IFN $\alpha$  responses to TLR7 stimulation. Furthermore, bone marrow-derived pDCs from mice with conditional ER $\alpha$  knock-out exhibited impaired capacity to produce IFN $\alpha$  in response to TLR7 engagement and significantly less *IRF5* mRNA expression than pDCs from WT mice. Taken together, our results dissect a novel mechanism by which differential regulation of IRF5 between males and females drives higher TLR7-mediated IFN $\alpha$  production in females. Our study therefore identified IRF5 as an attractive target for specific modulation of the IFN $\alpha$  pathway.

### **2. Contribution of IRF5 isoforms and polymorphisms to its role in the transcription of IFN $\alpha$**

Conflicting results regarding the role of IRF5 in IFN $\alpha$  transcription have been described and may account for cell type and stimulant specificities<sup>83,84,109,110</sup>. We showed that IRF5 levels prior to stimulation in primary human pDCs were correlated with the percentage of IFN $\alpha$ -secreting pDCs after two hours of TLR7 stimulation. To clarify the causal link, we utilized a novel vector-free delivery system to incorporate exogenous recombinant IRF5 protein into human primary pDCs. Unspecific effect of recombinant IRF5 protein was addressed by using another recombinant protein, TUBA1A, similar in size and origin to recombinant IRF5 protein, but irrelevant to the pDC IFN $\alpha$  pathway. As expected, experimental increase in IRF5 protein, but not in TUBA1A, enhanced the percentage of IFN $\alpha$ -secreting pDCs (Article 1, Figure 3C). In addition, in collaborative work with the group of JP Herbeval (CBNIT, University of Paris Descartes), we attempted to knock-down IRF5 expression in primary human cells using siRNAs. The three siRNAs tested had significant off-target effect on IRF7 (Figure D1A-B), in line

with the high sequence homology between the two IRF family members. This off-target effect prevented us to assess the individual IRF5 contribution to IFN $\alpha$  production by pDCs.

Cell type-specific differences in IRF5 function might related to cell type-specific expression of alternatively spliced isoforms of IRF5 <sup>56</sup>. Human pDCs express primarily V3 and V4 transcripts while T cells express the V4 and V1 transcripts <sup>56</sup>. Accordingly, we observed sex differences in IRF5 levels in pDCs but not T cells. Furthermore, we sorted pDCs from three patients and delivered two different recombinant IRF5 isoforms, V3 and V4 respectively using our standardized vector-free delivery protocol. Cells were then stimulated for two hours with CL097 and transcriptomic changes were analyzed on the Nanostring instrument. Given the small pDC numbers, enough material was recovered from only one study subject. This preliminary result (as from a single experiment) revealed that delivery of V3 and V4 lead to differential induction of IFN $\alpha$  subtypes as shown in Table D1. These preliminary result also suggest that the role of IRF5 in IFN $\alpha$  transcription is subtype-specific, in line with previous report <sup>91</sup>.

**Table D1: Fold change in normalized expression of IFN genes upon IRF5 recombinant protein delivery compared to control in sorted pDCs stimulated for 2hours with CL097**

	<b>IRF5 V3</b>	<b>IRF5 V4</b>
<b><i>IFNA2</i></b>	<b>0.5</b>	<b>37.5</b>
<b><i>IFNA4</i></b>	<b>1</b>	<b>2</b>
<b><i>IFNB1</i></b>	<b>31</b>	<b>123</b>
<b><i>IFNL3</i></b>	<b>3</b>	<b>4</b>

Inter-individual variability in IFN $\alpha$  production by pDCs is important with even more variability in females, in line with estrogen regulation of IFN $\alpha$  response. Furthermore, not all stimulated pDCs produce type I IFNs, in line with the concept of stochastic expression of IFN and ISGs <sup>67</sup>. It has been suggested that bimodal activity of STAT2 and IRF7 may be responsible for the stochastic nature of ISGs and IFN expression <sup>67</sup>. Interestingly, we found that basal IRF5 expression among pDCs is heterogeneous with some pDCs expressing no or low levels of IRF5, suggesting that IRF5 may also be subjected to bimodal expression. In contrast, we usually observed less heterogeneity when it came to IRF7 expression in pDCs. Nevertheless, increased heterogeneity in IRF5 expression may be attributed to the presence of specific isoforms. The antibody against IRF5 used in our study is recognizing all IRF5 isoforms but we cannot account for differences in the affinity for the various isoforms. Different isoforms of IRF7 also exist but alternative splicing variants may be produced at very low levels due to a premature stop codon in the mRNA, leading to nonsense-mediated mRNA decay <sup>464</sup>. It would be important to determine what threshold determines the “on/off” expression of IRF5 in pDCs.

Inter-individual variability may also be related to the large numbers of SNPs associated with pDCs IFN $\alpha$  production <sup>465</sup>. Of note, the consequences of given SNPs for pDC IFN $\alpha$  production depend on the stimuli used

but also on the cells surrounding pDCs during activation<sup>465</sup>. IRF5 polymorphisms have previously been associated to differences in IFN $\alpha$  response. The SLE-risk CGGGG-indel IRF5 haplotype is associated with increased IRF5 and IFN $\alpha$  levels in SLE patients<sup>101,104</sup>. Similarly, the SLE risk variant SNP rs4728142 located in the promoter region of IRF5 is associated with increased expression of the IRF5 gene<sup>466</sup>. We did not have access to data regarding IRF5 polymorphism for our study subjects. Recently, it was shown that SLE-risk CGGGG-indel IRF5 haplotype was associated with decreased IFN $\alpha$  production by pDCs from healthy donors in co-culture with B cells<sup>465</sup>. The authors suggested that the differential impact of IRF5 polymorphisms on IFN $\alpha$  production between SLE patients and healthy donors may be related to other gene polymorphisms, epigenetic changes or cellular microenvironment in SLE patients that may interact with IRF5 expression and affect the final function of IRF5 in the IFN $\alpha$  response<sup>465</sup>. However, IRF5 expression levels were not measured. Importantly, no effect of IRF5 CGGGG-indel on IFN $\alpha$  was detected for pDCs alone or in cocultures with NK cells<sup>465</sup>. The authors further showed that association between IFN $\alpha$  production and SNPs in healthy donors depended on the combination of stimulated cell types. The same group has previously showed that B cells could enhance pDC-mediated IFN $\alpha$  production<sup>45</sup>. B cells express high levels of IRF5 and sex difference in IRF5 expression has been reported in murine splenic B cells<sup>467</sup>. This data suggest that sex differences in IRF5 B cells expression may also indirectly contribute to sex differences in pDC IFN $\alpha$  response. However, further investigations are needed to conclude on this matter.

### 3. How ER $\alpha$ may regulate IRF5 expression and potential role of X-chromosome

Although we show that *IRF5* mRNA expression was significantly reduced in mice with a conditional knock-out for ER $\alpha$ , the precise functional mechanisms by which ER $\alpha$  might regulate IRF5 in pDCs are unknown. As described in section V.1.2 of the introduction, ER $\alpha$  can mediate its effect through different mechanisms. ER $\alpha$ -mediated effects on IRF5 were independent of ER $\alpha$  expression levels, which do not differ between pDCs from females or males (Figure D1C,<sup>31</sup>). An ER $\alpha$  binding site has been identified using the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu>) at position 128561334-128561609 on human chromosome 7 which is 16,385bp upstream the *IRF5* gene (*IRF5* chr7:128577994-128590088), suggesting that IRF5 may be regulated at the transcriptional level directly by ER $\alpha$  binding to ERE. Nevertheless, one of the described *IRF5* polymorphism, the CGGGG indel, is associated with increased expression of IRF5 itself due to the presence of an additional SP1 binding site<sup>101</sup>. As SP1 is a well-known binding partner to ER $\alpha$ , ER $\alpha$  may also be tethered on IRF5 promoter and not directly interacting with IRF5 DNA sequence. Chromatin immunoprecipitation (ChIP) enables to define the interaction of factors with a given chromosomal site in living cells through formaldehyde-mediated crosslinking of proteins to DNA and antibody-mediated immunoprecipitation. Polymerase chain reaction (PCR) analysis of the immunoprecipitate with primers flanking the *cis*-element reveals whether a specific DNA

sequence is recovered in an immune-specific manner, that is to say whether the protein was bound to the site in living cells. CHIP assays usually require 1 to 10 million cells. Recent optimizations have allowed to decrease starting cell number to about 10,000 cells for the study of histone modifications rather than transcription factor binding<sup>468</sup>. Implementing CHIP assays using pDCs will likely be challenging given the low cell numbers. To our knowledge, only one study performed CHIP on primary pDCs. Kuo and colleagues combined  $1 \times 10^5$  pDCs from five donors ( $5 \times 10^5$  pDCs total) to quantify H3K4 trimethylation at the *IRF7* promoter and IRF7 binding at the *IFNA10* and *IFNB* promoters<sup>469</sup>. The question of ER $\alpha$ -tethering to *IRF5* promoter by partner transcription factor could be addressed by Re-ChiP<sup>470</sup>, where ChiP with partner transcription factor (TF) will be followed by “release” of the TF-enriched chromatin and subsequent ER $\alpha$  ChiP. Nevertheless, the partner TF will have to be previously identified. Candidates, other than SP1, should be considered. Furthermore, the assignment of the distal ER $\alpha$  binding site identified in the ENCODE database (about 16 kbp upstream the *IRF5* gene) to *IRF5* gene regulation is more complicated. It is now well recognized that ER $\alpha$  frequently acts as long-range enhancers rather than classic transcription factors<sup>471</sup>. The chromosome conformation capture (3C) technique allows observing physical interactions between any pair of selected genomic loci<sup>472</sup>. Using the 3C method, chromatin segments that are in close spatial proximity are cross-linked, digested by a restriction enzyme and ligated. Ligation products are then detected and quantified by qPCR. The derived CHIP-3C method enables to ask whether specific proteins such as ER $\alpha$  are involved in long-range chromatin interactions<sup>473</sup>. One could use one primer from the distal ER $\alpha$  binding site and the other from *IRF5* promoter in qPCR on CHIP-3C material to see if ER $\alpha$  spatially links those two regions. Hence, new CHIP-based techniques may provide critical information on the mechanisms of ER $\alpha$  regulation of immune responses.

Furthermore, ER $\alpha$  could also affect IRF5 levels indirectly via modulation of IRF5 mediators. miR-146a is negatively regulated by estrogen<sup>474</sup> and targets IRF5<sup>475</sup>. miR-146a is induced<sup>476</sup> upon TLR7 and TLR9 activation in a cell type-specific manner<sup>70,71,475</sup> and negatively regulates IFN $\alpha$  production in pDCs<sup>70</sup>. It downregulates TRAF6, IRAK1 and STAT1 expression<sup>71</sup> and is expressed at lower levels in SLE patients than in healthy donors<sup>475</sup>. Given the intimate relationship between steroid signaling and immune responses, it is unlikely that IRF5 is the sole factor mediating sex differences in pDC IFN $\alpha$ . So far, expression of miR-146a has not been compared between male and female donors but miR-146a is an interesting candidate that could impact secondary IFN $\alpha$  production by pDCs in a sex-specific manner.

Finally, the ER $\alpha$ <sup>KO</sup> mice models used in our study do not exclude that non-hormonal pathways and particularly X-chromosome linked factors participate in sex-specific regulation of IRF5 in pDCs. Recently, it was shown that X chromosome dosage contributed independently from sex hormones to the sex bias in the pDCs TLR7-mediated IFN $\alpha$  response<sup>31</sup>.

#### 4. Potential role of IRF7

In our study, we did not find any sex-based difference in IRF7 protein expression and therefore focused on IRF5. Nevertheless, we did not exclude that differences in the pattern of activation of IRF7 may participate in the sex differences observed. We compared the kinetics of IRF7 phosphorylation upon TLR7 stimulation in females and in males (Figure D1D). Phosphorylation of IRF7 seems to peak earlier in females (20min) than in males (30min) but normalizes at 60min equally in females than in males. BTK and IRAK1, two kinases involved in IRF7 activation<sup>17</sup>, are X-linked and may display some degree of escape from X inactivation<sup>476</sup>. We therefore hypothesized that faster IRF7 phosphorylation in females may be related to increase expression of X-linked kinases. We compared IRAK1 and BTK expression at baseline in pDCs from five female and five male healthy donors. Our preliminary data suggest that there is no sex difference in their expression (data not shown). Furthermore, estrogens are known to modulate the PI3K/mTOR pathway, which is also involved in IRF7 activation. Importantly, IRF5 and IRF7 activation involves several common molecules. Better characterization of IRF5/7 activation, notably of its phosphorylation, may uncover sex differences in IRF5/7 activation.

#### 5. Unresolved questions on sex differences in pDC IFN $\alpha$ response and requirements of pertinent models for further studies

In our study, we uncovered a novel mechanism underlying sex differences in pDCs IFN $\alpha$  response but relevant questions remain opened. ISGs can be divided in different subclasses. First, antiviral ISGs, such as Mx1 or PKR, are highly sensitive to IFN induction, requiring only picomolar concentrations of type I IFNs. In contrast, “immunomodulatory” ISGs, such as IL-6, CXCL11, and TRAIL requires 100-fold higher IFN concentrations<sup>62</sup>. Their induction also requires prolonged signaling duration as well as high IFNAR concentration on the cell surface<sup>118</sup>. Quantitative differences in the expression of ISGs between males and females have been reported<sup>162</sup>. Qualitative information are missing but may provide critical insight on the link between increased IFN $\alpha$  production and faster disease progression in HIV-1 infected women<sup>236</sup>. Similarly, qualitative differences in IFN $\alpha$  subtypes production between males and females have not been studied.

Identification of the mechanisms underlying sex differences in immune responses is complex, especially the context of the different effects of sex hormones. The literature on the effect of sex hormones on pDC IFN $\alpha$  response has been conflicting. In particular, *in vitro* stimulation by sex hormones is poorly reproducible. Berghöfer et al. reported no effect of increasing of E2 concentrations (0.5-500nM) on TLR7-mediated pDC IFN $\alpha$  production<sup>289</sup>. CpG- and VSV-mediated pDCs IFN $\alpha$  production may be inhibited by progesterone<sup>477</sup>. Furthermore, pretreatment of female adult pDCs with dihydrotestosterone decreased TLR7-stimulated IFN $\alpha$  but not TNF $\alpha$  production, in a dose dependent manner<sup>478</sup>. Importantly, regular RPMI medium contains phenol red, which has an estrogenic effect and fetal bovine serum needs to be charcoal-treated to remove steroids. Hence, *in vivo* models appear more appropriate to the study of the effect of sex hormone on immune responses. *In vivo*

models agreed on the positive role of E2 on TLR-mediated pDC IFN $\alpha$ <sup>259,479</sup>. Nevertheless, *in vivo* exposure to physiological, supraphysiological, or pregnancy levels of E2 can abolish cycling of E2 levels that are potentially important to immune cell biology. Elevated systemic levels of E2 can profoundly deplete populations of hematopoietic progenitors which leads to alterations in number and phenotype of lymphocytes and DC in bone marrow and spleen. Estrogens exert a suppressive effect on both B and T lymphopoiesis<sup>252</sup>. Major breakthroughs were made possible by the use of ER knockout mice models, including a conditional model allowing for study of effect of ER $\alpha$  on specific cell types via the use of the Cre/LoxP system<sup>480</sup>. Sex differences have been reported for multiple viral diseases<sup>235</sup>, including influenza A virus and hantavirus. It would be of great interest to see if common mechanisms, potentially involving pDC IFN $\alpha$  response and ER $\alpha$ , underlie those reported differences.

## **II. Altered IFN $\alpha$ responses and increased inflammation impacts HCV disease severity in ART-treated HIV-1-HCV co-infected patients**

The second part of my thesis work consisted in linking specific alterations in pDCs IFN $\alpha$  pathway with pathological outcomes in HCV-HIV-1 co-infection

### **1. Results obtained**

Our study shows that HCV-HIV-1 co-infected individuals with minimal to moderate fibrosis exhibit increased inflammation levels than HIV-1 mono-infected individuals, under suppressive ART and with minimal impairment in the CD4<sup>+</sup> T cell compartment. We also report increased T cells and pDCs exhaustion in HCV-HIV co-infection but no difference in cellular immune activation. Importantly, stronger ISGs expression in pDCs from HCV-HIV-1 co-infected patients was significantly correlated to different measures of fibrosis, including Fibroscan values, ALAT levels and APRI score.

The impact of HCV on T cell activation in HCV-HIV-1 co-infected individuals is not clear, with contradictory data available<sup>412,413,415,481</sup>. Methodological differences, including in the inclusion groups can partially explain some of these discrepancies. In our study, T cell activation was measured on fresh whole blood. Consistently, we reported lower percentage of CD38+HLADR+ CD8+ in all groups than in studies using PBMCs<sup>415</sup>. Nevertheless, the percentage of CD38+HLADR+ CD8+ was higher in HIV-1 infected patients under suppressive ART than in healthy controls, in line with persistent impairment with ART. Co-infected patients displayed increased exhaustion/immunosenescence on CD8+ T cells, in accordance with previous report<sup>415</sup>. The percentage of CD57+CD8+ was negatively correlated to the CD4/CD8 ratio in HIV-1 infected individuals irrespective of their HCV status, in line with previous observation in HIV-1-infected individuals on suppressive

ART and in vertically HIV-infected children and adolescents on ART <sup>276,482</sup>. Interestingly, the percentage of CD57+CD8+ was also correlated to the absolute count of peripheral pDCs, which may be linked to type I IFNs-mediated generation of immunosenescent cells <sup>424</sup>. pDCs have been described in the livers of HCV patients, including histologically <sup>316 317</sup> and loss of circulating DC is associated with the histological grade of liver inflammation <sup>316</sup>. Accordingly, we found significantly fewer circulating pDCs in co-infected patients with severe fibrosis than in patients with mild to moderate fibrosis. pDCs were also more exhausted in co-infected patients as observed by LAG3 expression. LAG3-expression pDCs can negatively regulate T cell activation <sup>53</sup> but we did not find a correlation between pDC LAG3 expression and T cell activation. It is possible that generalized exhaustion results from the higher inflammation observed in HCV-HIV-1 co-infection.

Increasing evidences are pointed towards a role of persistent defects on innate immune responses and associated inflammation in morbidity and mortality in ART-treated HIV-1-infected patients <sup>139,483</sup>. IP-10 was the cytokine the most up-regulated in HCV-HIV-1 infected patient compared to HIV-1 mono-infected patients in our study. We observed a decrease in IP-10 levels at 12 weeks post-treatment completion, suggesting that inflammation may rather be driven by HCV replication.

## **2. Biological interpretation of the association between ISGs and fibrosis severity**

We suspect that the association between ISGs expression in pDCs and the extent of fibrosis reflects a true biological mechanism, as pDC ISGs levels were associated to three independent measures of fibrosis. Although expression of all individual ISGs was correlated to at least one measure of fibrosis, EIF2AK2 expression exhibited the strongest correlation to all three measures of fibrosis (Article2, Supplementary Table 5). EIF2AK2 expression in pDCs was not distinct from the expression of the other ISGs (Article2, Supplementary Table 4). Interestingly, HCV may hijack the antiviral EIF2AK2 pathway via EIF2AK2 phosphorylation to inhibit ISGs translation <sup>484</sup>, therefore preventing antiviral response. EIF2AK2 may thus be more relevant than the other ISGs.

Higher fold change expression to healthy controls in the co-infected group with more advanced fibrosis may highlight a role of fibrosis-driven inflammation in the enhancement of ISGs expression in pDCs. Nevertheless, plasma cytokine levels were not increased in patients with more advanced fibrosis than in patients with mild to moderate fibrosis. These results should be considered carefully given the small patients numbers for this group (n=4). Conceivable, strong ISGs expression may represent a surrogate marker for exposure to type I IFNs, although it is not clear why stronger ISGs expression was only observed in pDCs and not in PBMCs. Similarly, the cytokine that had the most prominent change between co-infected and HIV-1 mono-infected patients, IP-10, is known to be upregulated by type I IFNs. Therefore, increased ISGs expression and inflammation in HCV-HIV-1 co-infection may be orchestrated around increased IFN $\alpha$  levels, even if the difference did not reach significance. Chronic exposure to type I IFNs could promote liver fibrosis by several mechanisms including dampened IFN $\gamma$  production by NK and CD8+ T cells, induction an antagonist form of IP-10

preventing hepatic recruitment of anti-viral NK and CD8+ T cell effectors<sup>384</sup>. pDC-derived IFN $\alpha$  may promote liver injury after initial damage of hepatocytes, by inducing apoptotic pathways in hepatocytes<sup>446</sup>.

As mentioned earlier, it is unclear why strong ISGs expression is observed in pDCs but not PBMCs in co-infected patients but may be related to type III IFN signaling. Supplementary unpublished findings were obtained in our study of HCV-HIV-1 co-infection. These findings were excluded from our manuscript since the type III IFN genotype were not yet available for all patients included. We found that, in pDCs, IFNAR1 expression, although not correlated to individual ISG expression (Figure D2), was surprisingly negatively correlated with ISG score ( $p=0.032$ ,  $R^2=0.28$ , Figure D2B), defined by the average expression of E12AK2, Mx1, ISG15 and IFI44. In contrast, in PBMCs, IFNAR1 expression is positively correlated to E12AK2 ( $p=0.0009$ ,  $R^2=0.51$ , Figure D2C). It has been previously suggested that IFNAR1 expression in HCV infected patients is dependent on IL28B rs12979860 genotype, with a direct correlation with endogenously produced IFN $\lambda$  in C/C-individuals<sup>485,486</sup>. So far, we were able to collect IL28B polymorphism (rs12979860) for only eight patients: three were CC, three C/T and two T/T. In Europeans, the favorable C/C haplotype is the major *IFNL3* allele while it is the minor one in Africans. Accordingly, the two T/T-individuals in our study were from African and North African origins. In these preliminary results, IFNAR1 expression in pDCs did not appear to differ according to IL28B genotype (Figure D2D). Interestingly, IL28B polymorphisms have been associated to ISGs expression levels<sup>376</sup> and to liver fibrosis<sup>487</sup>. Of note, the effect of IL28B polymorphisms on liver fibrosis was shown to be more important in early stages of fibrosis (F0-F2) than in late and more severe stages (F3-F4)<sup>487</sup>.

Type I and III IFNs induce similar pattern of ISGs although with different kinetics<sup>326</sup>. Given that pDCs respond to IFN $\lambda$ 3 while other major PBMCs subsets are largely unresponsive to type III IFNs<sup>355</sup>, it is tempting to hypothesize that the opposite direction of the correlation between IFNAR1 mRNA and ISGs in pDCs and PBMCs may be related to cell-type specific effect of IFN $\lambda$ . The strong levels of ISGs observed in HCV-HIV-1 co-infected individuals may therefore be related to both type I and III IFNs signaling pathways. Nevertheless, we did not observe any difference in IFN $\lambda$ 3 mRNA in pDCs from our four patient groups. IFN $\lambda$ 3 mRNA was not detected consistently in all samples. In an effort to better characterize the potential contribution of type III IFNs, we plan to measure plasma levels of type III IFNs.

### **3. Clinical relevance of our findings and limitations of our study**

Our study has several limitations. Our findings are restricted to male sex and HCV genotype 1. As per our patient selection strategy, the HCV-HIV-1 co-infected individuals recruited in our study displayed minimal to moderate fibrosis. Furthermore, fibrosis is a long process while our measure of ISGs levels was at a given time point and may be subject to over-time fluctuations. Stronger associations between ISGs levels and fibrosis score could be highlighted by a longitudinal study. Finally, the role of type III IFNs was not sufficiently studied. Furthermore, we did not include a HCV mono-infected group. Comparison between HCV-HIV-1 co-infected and

HCV mono-infected patients would help uncover whether altered ISGs expression and increased inflammation is also occurring in HCV mono-infection, therefore being independent of HIV-1-mediated immune alterations. Higher inflammation markers in HCV-HIV-1 co-infected individuals may skew the robustness of predictors fibrosis, normally used in HCV mono-infected individuals<sup>488</sup>. Besides, it could have provide meaningful insights on the relevance of current treatment guidelines: Whether common guidelines for HCV-HIV-1 co-infected and HCV mono-infected patients are justified and whether it may be relevant to treat earlier HCV mono-infected patients with minimal fibrosis in line with inflammation levels. HCV direct-acting antiviral (DAA) therapy agents have been recently licensed and have lead to tremendous progresses in the treatment of HCV in terms of both efficacy and toxicity profiles. Both clinicians and patients are waiting for IFN-free DAA treatments. Nevertheless, high costs remain a major hindrance, notably given that treatment for HIV-1-HCV co-infection often occurs in resource-constrained settings. Clinicians may treat HCV-HIV-1 co-infected cirrhotic patients but defer HCV therapy for those with early-stage fibrosis until generic HCV protease inhibitor are available. Furthermore, in the new era of IFN-free DAA treatments, clinical practice guidelines no longer differentiate between HCV-HIV-1 co-infected and HCV mono-infected patients and recommend only to treat patients with significant fibrosis<sup>489</sup>. Excessive inflammation is associated with the occurrence of non-AIDS defining events<sup>139</sup> and favors HCV-mediated liver damage<sup>384</sup>. Our study provides a rationale for treating HCV-HIV-1 co-infected individuals, even with mild fibrosis, as early as possible, and despite the high costs of DAA.

### **III. Relevance of pDC-derived type I IFNs as therapeutic target for HIV-1 infection**

pDC type I IFNs responses are more and more referred to as disease-promoting agents. I will now discuss more generally the relevance of pDCs type I IFNs responses in immune responses. Then, I will provide some general idea about potential application-focused therapeutic strategies to limit IFN $\alpha$  production in HIV-1 infection, with a focus on targeting IRF5.

#### **1. Contribution of pDC-derived IFNs in immune responses**

##### **1.1. Evolutionary perspectives**

Studies investigating evolution of type I IFN and TLRs signaling pathways across species highlighted their crucial importance. It has been speculated that a complete IFN-inducing antiviral system was established after the shark species in evolution<sup>490</sup>. The maintenance of numerous IFN $\alpha$  subtypes throughout evolutionary history strongly suggest a survival strategy, with specific type I IFN subtype potentially capable of inducing subsets of ISGs that are effective at inhibiting different viruses<sup>66</sup>. In parallel, the TLR signaling pathway is highly conserved in structure and function from insects to vertebrates<sup>491,492</sup>. Most, but not all, vertebrates have exactly one gene orthologous to each of the TLR2-5 and TLR7-9 subfamilies<sup>493</sup>. Multigene families of the immune system usually

evolved coincidentally but this is not the case for TLRs<sup>493</sup>. Selective pressure for maintenance of specific PAMP recognition dominated the evolution of most of TLRs<sup>493</sup>. Specific receptor may therefore be preferentially subject to evolutionary changes due to interaction with given “external” environment<sup>492</sup>. TLRs and their downstream molecules co-evolve under strong purifying selection<sup>492</sup>, although downstream genes usually evolve faster than upstream genes. The more distant is their position to the receptor, the less selection pressure is applied on downstream genes in the TLR pathway<sup>492</sup>. Overall, the data suggest that the evolution of type I IFNs and TLRs signaling pathways is dictated by pathogens threats to given species.

At the human species level, stronger purifying selection applies to endosomal TLRs than to extracellular TLRs between different populations<sup>494</sup>. Stronger selection pressure applied to endosomal TLRs<sup>494</sup> may be related to the tighter PAMPs diversity of viruses, usually recognized through nucleic acids, as well as autoimmune avoidance as duplication of the TLR7 gene renders mice prone to develop autoimmunity<sup>495</sup>. In opposite, the higher evolutionary flexibility of the cell surface TLRs may allow for some TLRs mutations to be positively selected either worldwide or in a population-specific manner<sup>494</sup>. Patients with UNC93B deficiency, unresponsive to stimulation of endosomal TLR stimulation, are predisposed to herpes simplex virus-1 infection<sup>496</sup>. Similarly, the group of Jean-Laurent Casanova showed that a single-gene inborn error in *IRF7* gene can underlie life-threatening influenza infection in humans<sup>497</sup>. Surprisingly, the IRF7-deficient child displayed a narrow infectious phenotype, restricted to severe influenza, and was protected by annual influenza vaccination<sup>497</sup>. This case study suggests that IRF7 may not be necessary to mount vaccine-induced protection against influenza. Given the crucial role of endosomal TLRs in antiviral responses, it is surprising that such deficiencies do not lead to more severe viral infections. It has been hypothesized that genes, required for protective immunity in the past, may become redundant in the context of improved social, hygiene, and healthcare in modern society<sup>498</sup>. Nevertheless, patients with impaired STAT1 mediated IFNAR signaling are suffering from lethal viral diseases<sup>498</sup>. Furthermore, patients bearing genetic mutations in MyD88 and IRAK4 suffer from bacterial infections but not from life-threatening viral infections<sup>498</sup>, although those mutations also impair endosomal TLRs. Improved hygiene and healthcare may therefore not be the only factors responsible for the protection against virus in the aforementioned patients but signaling redundancy may also play a critical role. As discussed below, antiviral response may be mounted by cells expressing endosomal TLRs but also by infected cells via other PRRs such as RLRs. RLRs and particularly RIG-I, are under strong selective pressure imposed by specific viruses<sup>499</sup>. Altogether, these evolutionary data suggest that immune protection against viruses can be mediated by PRRs other than endosomal TLRs when the later have defective signaling. In that context, non-pDCs may contribute to immune protection.

## **1.2. Respective role of pDCs and non-pDCs cells in type I IFN production during viral infection**

While pDCs are known as the professional type I IFN producing cells, most immune cells are capable to produce IFNs. It is therefore important to consider the contribution of both pDCs-derived type I IFNs and non-pDCs derived IFNs. A recent review concluded that IFN production by uninfected pDCs acts as failsafe mechanism, activated only when viral infection gets systemic whereas IFN production by infected cells serves as first line of defense to block viral replication at its portal of entry in the body<sup>498</sup>. Indeed, non-pDCs cells have been shown to be the major source of IFN production upon local infections by several viruses<sup>498</sup>. In the case of pulmonary infection by Newcastle disease virus (NDV), type I IFNs are produced in the lungs by infected alveolar macrophages and not lung pDCs<sup>500</sup>. However, in the absence of viral recognition by alveolar macrophage, that is to say when the first defense line is broken, pDCs produce IFN $\alpha$  (Kumagai et al., 2007). Similarly, pDC-derived type I IFNs are important for systemic but not local HSV infection in the vaginal tract<sup>498</sup>. The contribution of pDCs among type I IFN producing cells in response to HSV depends on route of infection<sup>498</sup>. The site of infection seems therefore to influence pDC IFN $\alpha$  response. Whether it is related to the intrinsic nature of the site of infection, viral tropism, or to specific viruses, is still unclear. Data on the role of pDCs in *in vivo* models are still limited. Type III IFNs are involved in the control of epitheliotropic viruses such as HSV in a non-redundant fashion with type I IFNs while type I IFNs are responsible for the control of systemic infections<sup>501,502</sup>. It is therefore tempting to speculate that inhibition of pDCs IFN production at mucosal barriers may be related to type III IFNs signaling<sup>498</sup>.

Importantly, contribution of non-pDCs to virus-induced type I IFNs production during systemic infections is increasingly recognized<sup>498</sup>. In systemic NDV infection, type I IFNs are also produced by cDCs, macrophages in the spleen, monocytes and macrophages in peripheral blood<sup>500</sup>. Uninfected XCR1+ DC can produce large amounts of type III IFNs and IFN $\beta$  in response to HCV or dsRNA stimulation, irrespective of their own infection<sup>498</sup>. Sizing this contribution is difficult as, although non-pDCs produce up to 10 times less type I IFN on a per-cell basis, their higher frequency may increase their total contribution. However, *in vivo* experiments of pDC depletion during systemic viral infections showed a reduction of over 95% in type I IFN production<sup>503</sup>. Spleen appears to be an important organ for type I IFN production in response to blood-borne pathogens<sup>500</sup>, with splenic pDCs being prone to high levels IFN production<sup>498</sup>. It would be of interest to further characterize the impact of the confinement of pDC-derived type I IFNs in the spleen to type I IFN-driven pathological effects.

One major feature of pDC IFN production is the lack of requirement of infection. pDCs are therefore able to promote protection even against viruses interfering with the signaling pathway leading to IFN production. Hence, the group of Shizuo Akira showed that Sendai virus (SeV) with mutated C proteins but not WT SeV triggered production by murine alveolar macrophages *in vivo*<sup>500</sup>. C proteins disrupted type I IFN responses by alveolar macrophage. In contrast, pDCs produced IFN upon intranasal infection of WT SeV in mice. Antiviral action of pDC-derived IFNs may still be limited in infected cells if the virus hijack IFNAR signaling but will protect uninfected cells from infection and orchestrate innate and adaptive immune responses.

Altogether, these data show that pDCs-derived type I IFNs may be dispensable for the control of certain

local infections in line with IFN production by infected cells. pDCs type I IFNs responses are consistently activated upon systemic infections as their potential at controlling the infection outweighs the risk for type I IFNs-mediated immunopathology. If viral persistence is established, pDCs type I IFNs then fuels immunopathology such as in chronic HIV-1 infection.

## **2. Potential of type I IFN inhibition in HIV-1 therapeutic strategies**

Type I IFNs have been suggested to play a detrimental role in chronic HIV-1 infection. Although therapeutic interventions targeting IFN $\alpha$  to prevent excessive immune activation and inflammation have been initiated, the repercussions remain rather unpredictable. Various factors have to be considered for therapeutic modulation of type I IFN signaling, including (1) timing and duration of delivery, (2) local quantity/concentration of antagonists and (3) nature of targeted cells. In this section, I will discuss potential therapeutic strategies to block of type I IFNs in chronic HIV-1 infection that could help minimizing undesirable repercussions. Let me start by highlighting the importance of the timing of such therapeutic approach.

### **2.1. Timing and duration: Key consideration for therapeutic blockade of type I IFNs in pDCs in HIV-1 infection**

As discussed earlier, the role of type I IFNs is dichotomous in HIV-1 infection, with rather protective effects in acute infection but rather deleterious effects in chronic infection. The group of Daniel Douek showed that a four-weeks treatment with an IFNAR antagonist during the the acute phase of infection resulted in delayed peak expression of antiviral ISGs and attenuated T cell activation in lymphoid tissue yet accelerated CD4+ T cell depletion in the blood and LNs and progression to AIDS, in rhesus macaques<sup>233</sup>. This suggests that, *in vivo*, IFN $\alpha$  effect on cell intrinsic antiviral state may outcome pDC/IFN $\alpha$ -mediated effect on viral dissemination during the acute phase of infection. In contrast, continued administration of IFN $\alpha$ 2 after systemic dissemination of the virus lead to an IFN desensitized antiviral state. Continued administration was also associated with greater CD4+ T cell depletion compared to placebo, in line with the deleterious IFN $\alpha$ -mediated TRAIL killing of CD4+ cells. Therefore, I would speculate that antiviral functions of IFN $\alpha$  but not its immunomodulatory functions might be subject to desensitization, the latter being responsible for SIV/HIV-1 pathogenesis. Overall, these data emphasize the importance of the timing and duration of IFN-induced innate responses in regard of SIV/HIV-1 overall disease course and argue against therapeutic strategies to block type I IFN signaling in acute infection<sup>233</sup>. No argument is provided here against applying such strategy in chronic HIV-1 infection.

### **2.2. Targeted blockade of type I IFNs action**

IFN $\alpha$  exerts multiple effects on different cells. Studies using systemic blockade of type I IFN have consequently showed the difficulty to predict outcomes *in vivo*<sup>233</sup>. Local delivery of antagonists may limit their

effects. However, except maybe in LN, local delivery makes little sense in HIV-1 infection. Practically, the wide distribution of LN throughout the body renders local delivery very challenging. Targeting a specific cell type may also limit uncontrolled effect. Innovative immunotherapeutics, termed immuno-IFN, have recently been designed. Based on a covalent association between mutated type I IFN with decreased affinity for its receptor and an antibody with high avidity for a molecule specifically expressed on target cell types<sup>498</sup>, immune-IFN prevent off-target effects. The use of IFNs with close to maximal potency mediated strong off-target effects despite their coupling to cell-type specific antibodies and/or their local delivery <sup>498</sup>. Importantly, similar strategies could be used for targeted blockade of type I IFN signaling. An IFN $\alpha$ 2 carrying a single amino acid substitution has been shown to form a binary complex with IFNAR2 devoid of any type I IFN activity as it cannot bind IFNAR1 <sup>498</sup>. Thus, this type I IFN antagonist can be similarly coupled to an antibody specific for a cell surface marker and create an immuno-IFNant. Targeting immuno-IFNant to CD4<sup>+</sup> T cells may be a viable option. It may prevent TRAIL-mediated killing and exhaustion of those cells. So far, data have been generated exclusively *in vitro*. The efficiency and tolerability profiles of immune-IFNantagonist need to be validated *in vivo*.

### 2.3. Functional blockade of pDCs

During my PhD project, I have identified IRF5 as a potential target to decrease IFN $\alpha$  production in HIV-1 infection. Based on the transcriptomic data generated in my second project on HCV-HIV-1 co-infection, we can observed that, at the mRNA levels, IRF5 expression does not differ between ART-treated chronically HIV-1 infected and healthy donors but exhibits a significant inter-individual variability (Figure D1E). Hence, it is possible to reduce IRF5 levels in HIV-1 infected individuals and even to remain within physiological levels (by aiming at levels displayed by the lower group of donors). Which strategies could we then use? First, it will be important to identify an antagonist, by for instance screening for IRF5 chemical inhibitors. Effects of chemical inhibitors will likely to be systemic. In line with its role as a pro-apoptotic factor, IRF5 may exhibit tumor suppressor function <sup>110</sup>. Consistently, IRF5 expression is absent or significantly decreased in immortalized tumor cell lines and primary samples from patients with hematological malignancies <sup>91,504</sup>. Loss of IRF5 tumor suppressor function may contribute to tumorigenesis as shown for breast cancer breast <sup>505</sup>. Partial or targeted abrogation of IRF5 expression may prevent such deleterious effect. Based on our study of physiological model of sex differences in pDC IFN $\alpha$  production, small reductions in pDC IRF5 expression can have significant impact on IFN $\alpha$  levels and thus on subsequent IFN $\alpha$ -mediated detrimental effects.

How could IRF5 expression be specifically targeted? I considered how pDCs could be genetically engineered to that purpose. Pioneer work in gene therapy comes from the cancer field. Early-phase clinical trials for cancer therapy are currently evaluating the potential of engineered T cells, either expressing heterodimeric TCR or chimeric antigen receptor, and have lead to promising results <sup>506</sup>. Autologous pDCs are difficult to use for

adoptive transfer owing to their scarcity. Nevertheless, pDCs can be generated *in vitro* from CD34<sup>+</sup> progenitor cells<sup>31,507</sup>. It has been shown that *in vitro* differentiated non-gene-modified pDCs can be adoptively transferred into mice to increase antiviral effect against RSV infection<sup>508</sup>. Importantly, the effect of adoptive transfer is dependent on the cell life span. The long life span of clonal T cells makes them suitable for adoptive immunotherapy<sup>506</sup>. In contrast, pDCs have a short lifespan so that the effect of their adoptive transfer will be limited. Long-term effect can be achieved through engineering of hematopoietic stem/progenitor cells (HS/PCs). Escobar and colleagues have elegantly provided proof of principle of a safer and more effective delivery strategy based on gene therapy<sup>509</sup>. Exogenous IFN $\alpha$  has been considered as anticancer treatment but was associated to substantial toxicity. Their aim was to force IFN $\alpha$  expression in tumor-infiltrating macrophages to induce various anti-tumoral effects. They built a lentiviral vector containing an IFN $\alpha$  transgene (1) under the control of the Tie2 enhancer/promoter to ensure expression in lineages derived from for HS/PCs and (2) associated to a miRNA-based off-switch control to prevent the expression of the transgene in HS/PCs. The miRNA-based off-switch control was based on miR-126 and miR-130a as those are expressed in the HS/PC compartment but not in mature blood cells. Interestingly, the authors showed that antitumor efficacy even at low chimerism of transduced cells, so that genetic modification of only a fraction of HSCs could be envisaged to further limit unwanted repercussions. Similar strategy could therefore be used to target IRF5 in pDCs from HIV-1 infected individuals by replacing IFN $\alpha$  transgene by a miRNA or siRNA targeting IRF5. The miRNA/siRNA should be chosen cautiously as sequence homology between IRF5 and IRF7 may trigger unwanted effects.

In theory, I believe that the strategy could be attractive. Let us now appreciate it from a clinical perspective. Importantly, safe and effective HSC gene transfer by lentiviral vectors has been evidenced in clinical trials of gene therapy for inherited monogenic diseases<sup>510,511</sup> and failsafe strategies can be implemented to eliminate gene-modified cells if they display unwanted effects. Such strategies are certainly more complicated and risky than stopping taking pills containing IRF5 agonist. In addition, it is noteworthy to precise that myeloablative chemotherapy or immunosuppression are used for successful incorporation of the genetically modified HSCs and for elimination "original" HSCs. One major concern regarding the implementation of such strategy in HIV-1 infected individuals is the additional immune depletion caused by treatment, which could amplify the preexisting immune deficit. Nevertheless, it has been shown that autologous stem cell transplantation in HIV-1 infected patients affected by relapsed or refractory lymphoma do not worsen initial immune impairment or enhance viral replication or peripheral HIV-1 reservoir in the long-term<sup>512</sup>. The toxicity of chemotherapy and the transplant procedure would imply several months of hospitalization and recovery for the patient. Would HIV-1 infected individuals with well-controlled infection subject themselves to such procedure to reduce their inflammation and immune activation while they are otherwise seemingly healthy? Most certainly not. In conclusion, strategies based on IRF5 chemical antagonist might be the best option and will have to be tested *in vivo*.

## 2.4. Models for *in vivo* validation

Carefully consideration of the animal model the most appropriate at the outset of studies evaluating therapeutic strategies is crucial. Humanized mice transplanted with human immune tissues or cells have been developed to study HIV-1 infection<sup>513</sup>. This model may have several advantages over the NHP model including costs, differences in MHC class I genotypes and the use of HIV-1 instead of SIV. HIV-1 infection in humanized mice results in T cell depletion and correlated with immune activation<sup>513</sup>. In addition, the development of functional human pDCs in lymphoid tissues has been reported in different humanized mouse models<sup>514</sup>. Notably, pDCs had adequate TLR7 responses in different models<sup>259,514</sup>. As the CD4+ T cell population declined and infection progressed, pDCs from the bone marrow developed impaired responses to TLR7 and TLR9, similar to what has been suggested in chronic HIV-1 infection in humans. One limitation of this model remains the limited number of human cells, which poses a major hindrance not only for the simultaneous comparison of human pDCs in the blood and lymphoid organs but also for longitudinal studies. Another limitation is the inability to fully reproduce complex and multi-faceted inflammatory process, as seen in HIV-1 infection in humans. Altogether, these initial reports are encouraging but the humanized mouse model may not yet be suited for testing strategies aiming reducing immune activation and inflammation in HIV-1 infection.

## 3. Therapeutic potential of type III IFNs

Type III IFNs may mediate antiviral response at local mucosal sites without inducing deleterious immune-mediated inflammation, observed for type I IFNs. Interest is therefore growing regarding the therapeutic potential of type III IFNs. Type III IFNs may act in a non-redundant fashion with type I IFNs in the gut<sup>501,502</sup> but some degrees of redundancy between type I and III IFNs signaling pathways have been noted *in vivo* for infections in the respiratory tract<sup>515</sup>. Such differences may be related to the nature of those two mucosal barriers. Indeed, the gut is associated with a higher risk of uncontrolled inflammation than the respiratory tract and therefore may require maintaining immune response locally and not involve systemic responses. A recent study using humanized liver chimeric mice showed that HCV replication may be controlled by type III but not type I IFNs in human hepatocytes<sup>516</sup>. Furthermore, it has been proposed that HCV antiviral effect of PegIFN $\alpha$  treatment may be mediated through type III IFNs signaling pathway. PegIFN $\alpha$ -mediating induction of IFN $\lambda$ R1 expression on hepatocytes may boost endogenous IFN $\lambda$ 3 effects<sup>378</sup>. Accordingly, pre-activation of the IFN pathway may be associated to the inability to further increase IFN $\lambda$ R1 expression, leading to refractoriness to treatment<sup>498</sup>. Taken together, these data suggest that IFN $\lambda$  therapy may be a valid therapeutic option in HCV infected patients, who did not respond to PegIFN $\alpha$  therapy and particularly in the context of HIV-1 co-infection.

Besides, type III IFNs can also mediate strong antiviral effects against respiratory viral infections when given prophylactically<sup>517,518</sup>. Reinfection after clearance of HCV infection can occur. Hence, type III IFNs could be given prophylactically in patients who are at risk for recurrent HCV re-infection. Furthermore, HCV reinfection after liver transplantation is well documented and disease progression is accelerated compared with the non-transplanted population<sup>519</sup>. Type III IFNs could also be used to prevent new liver infection<sup>520</sup>.

Given their limited deleterious effects, one could ask whether type III IFNs may be considered as therapeutic strategy for HIV-1 infection. Importantly, type III IFNs are only sufficient to protect against viruses that infect cell types responsive to type III IFNs<sup>118</sup>. Type III IFNs can inhibit HIV-1 infection of blood monocyte-derived macrophages that expressed IFN-lambda receptors<sup>521</sup> but are unlikely to mediate antiviral state in CD4+ T cells, the main targets of HIV-1. In conclusion, type III IFNs could be used to modulate the HCV-driven increased inflammation in HCV-HIV-1 co-infection observed in our study but may not impact HIV-1 replication. In the context of IFN-free DAAs-based treatments and their high costs, PegIFN $\lambda$  therapy could still be as primary therapeutic strategy for cost-effectiveness purposes, in a similar but maybe more relevant way as what as been proposed previously for PegIFN $\alpha$  treatment<sup>380</sup>.

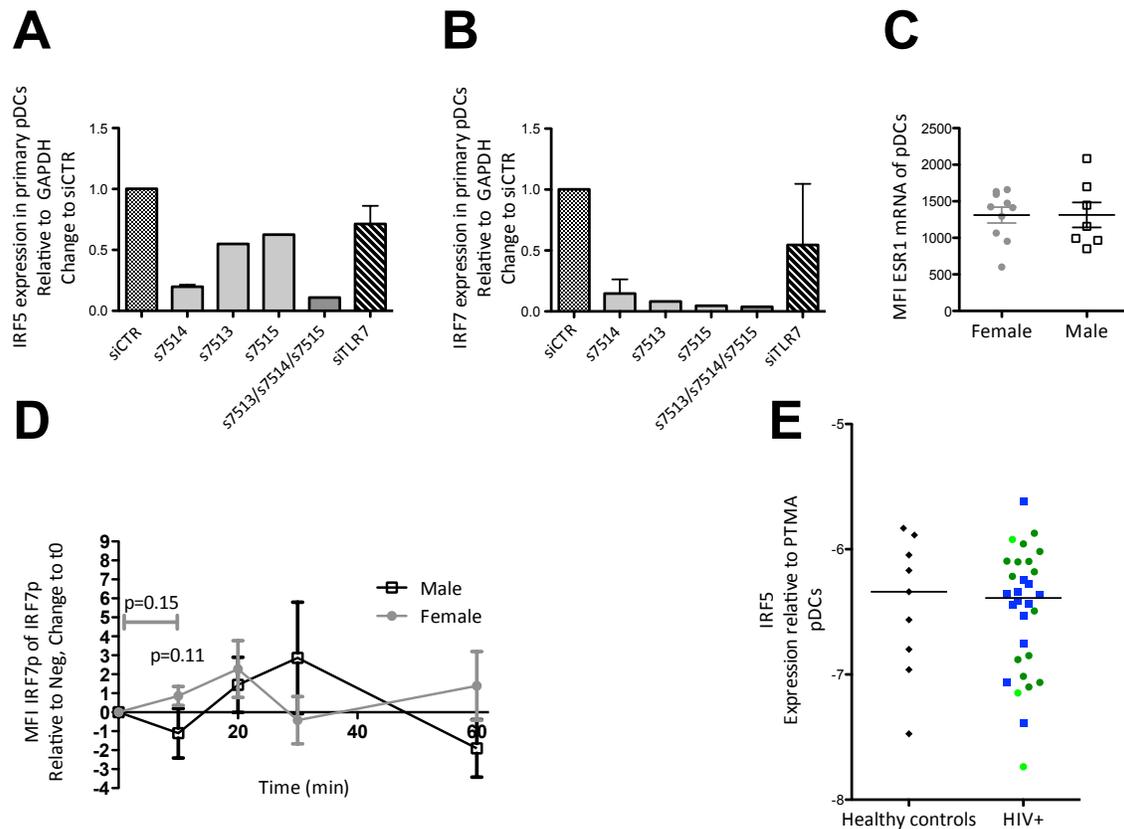
#### **IV. Open questions on pDCs and type I IFNs: What I would be interested in studying next**

Many parameters influence the pDC type I IFNs response. pDCs maintain constitutive low levels of IFN $\alpha$  as a priming mechanism allowing for production of high amounts of IFN $\alpha$ . The mechanisms involved in controlling endogenous IFN $\alpha$  levels remain poorly understood even if they have the potential to shape, indirectly though, subsequent pDC IFN $\alpha$ -mediated immune responses. I believe this is a major open question regarding pDC biology. During the preparation of this thesis I came across extensive literature on type I IFNs. Something that caught my attention was the relationship between commensal microbiota, IFN $\alpha$  priming and responses to viral infections in neonates. Studying pDC IFN $\alpha$  response in viral infections interests me deeply. Assessing differences between neonates and adults is somehow close to dissecting differences between males and females. It is fascinating how intrinsically linked are so many aspects of our biology such as our endocrine and immune systems or commensal microbiota with our immune system.

Increased susceptibility to infection in neonates has been linked to limited early type I IFN responses<sup>522</sup>. In addition, reduced pDCs IFN $\alpha$ -producing capacity has been reported in neonates compared to adults<sup>523,524</sup>. While reports agree on normal pDCs counts in neonates<sup>523</sup>, the mechanisms underlying such impairment remain elusive but may include impaired IRF7 nuclear translocation or downregulation of critical antiviral genes in infant pDCs, including IRF7<sup>522</sup>. Hence, pDCs may not exert adult-like function *in vivo* in the early life microenvironment.

Interestingly, commensal microbial flora may participate in the basal IFN $\beta$  production of immune cells and therefore in the systemic calibration of type I IFN responses<sup>16,525,526</sup>. Similar mechanisms may be in place for the establishment of endogenous IFN $\alpha$  production by pDCs. The mammalian fetal intestine is essentially sterile and the first exposure to maternal microbiota occurs during passage through the birth canal during the first hours of delivery<sup>527</sup>. Major evolution of gut microbial community happens later at weaning and introduction to solid food<sup>527</sup>. It is tempting to speculate that reduced pDCs IFN $\alpha$  responses in neonates may be linked to a defect of IFN $\alpha$  priming by commensal microbial flora. In line with this hypothesis, it has been shown that children born by caesarean have atypical microflora colonisation compared to those born vaginally<sup>528</sup> and are more prone to hospitalisation for bronchiolitis<sup>529,530</sup>. Furthermore, respiratory syncytial virus (RSV) infection can cause bronchiolitis in infants and is associated with limited type I IFN and pDC responses in neonates<sup>531</sup>. Altogether, these data show that gut microflora play a part in the development of the infant immune system and that defective type I IFNs response in neonates is associated with viral infections. Further investigation of the interactions between commensal microbial flora and pDCs IFN $\alpha$  production is needed.

In conclusion, my thesis work highlights how IFN $\alpha$  production by pDCs impacts inflammation and immune activation. HCV-HIV-1 co-infection is associated with increased inflammation and strong ISG expression in pDCs associated with disease severity. IRF5 was uncovered as a target to modulate deleterious IFN $\alpha$  production based on physiological model of sex differences in pDC IFN $\alpha$  production. In theory, strategies to modulate IRF5 expression are viable but *in vivo* experiment are required to determine its therapeutic potential.



**Figure D1: Supplementary data on sex differences in TLR7/IFN $\alpha$  pathway in pDCs**

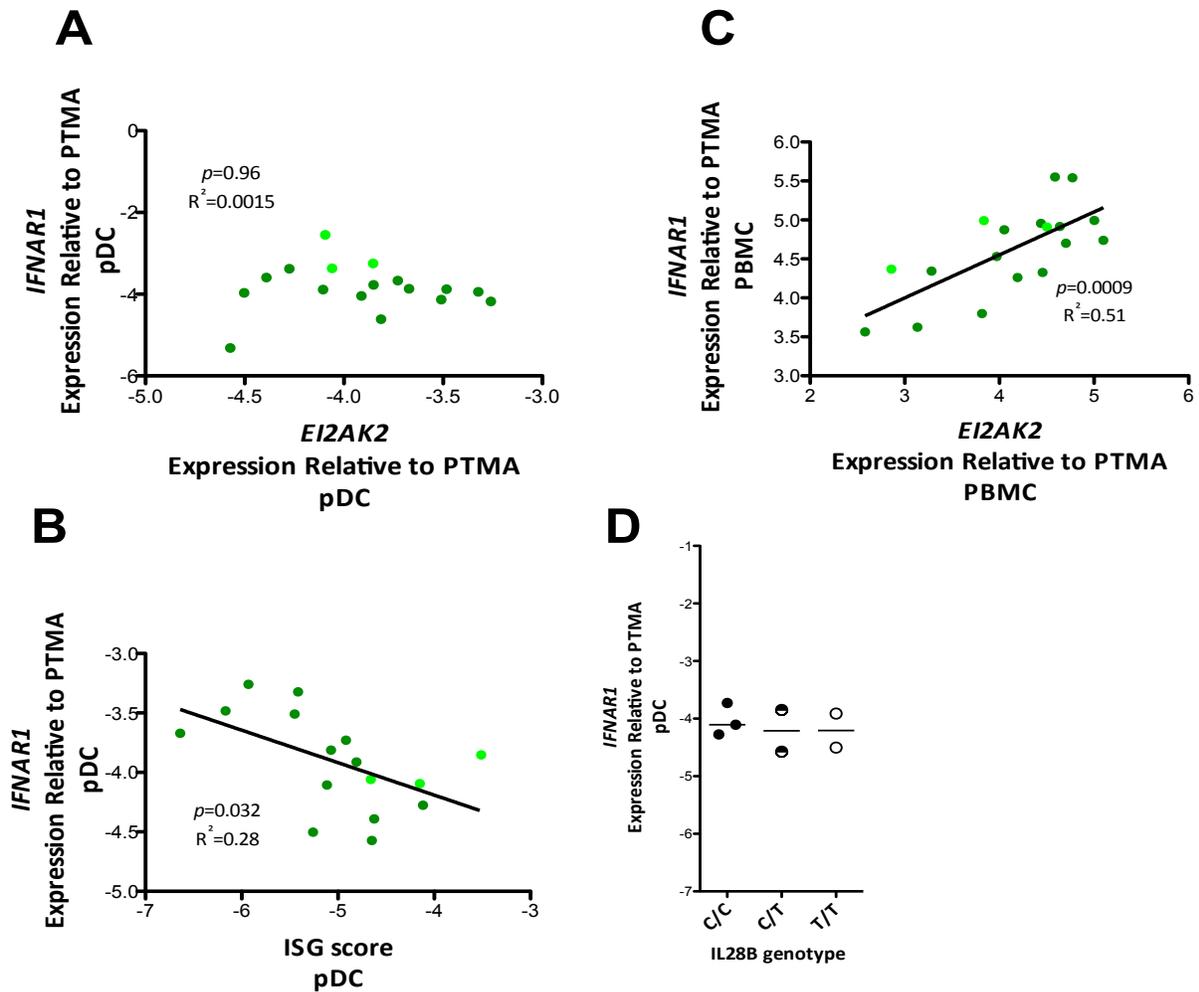
(A,B) Primary human pDCs were transfected with 160nM of siRNA using DOTAP as delivery vehicle. Different siRNAs against IRF5 were tested (s7513, s7514, s7515). siRNA against TLR7 (siTLR7) was used as additional control. After 24hours, IRF5 (A) and IRF7 (B) mRNA expressions were assessed by qRT-PCR. Expression was normalized to GAPDH. Change to scrambled siRNA (siCTR) is represented.

(C) ESR1 expression was measured in pDCs using the FlowRNA assay.

(D) PBMCs from healthy donors were stimulation by the synthetic TLR7 agonist CL097. The fold change in the expression level of phosphorylated IRF7 in pDCs was determined by phospho-flow cytometry.

Statistical significance between sex was determined by Mann-Whitney t test and statistical significance to time 0 by Wilcoxon Rank test.

(E) IRF5 expression was measured in 100 pDCs from 9 healthy donors and 30 ART-treated chronically HIV-1infected patients (HepACT-VIH cohort) on the BioMark HD. Expression levels were normalized to housekeeping gene PTMA. Blue squares represent HIV-1 mono-infected individuals. Rounds represent HCV-HIV-1 co-infected individuals with mild to moderate fibrosis (dark green) or with severe fibrosis (light green).



**Figure D2 Association between ISGs levels, IFNAR1 expression and IL28B polymorphism in HCV-HIV-1 co-infected patients**

Expression of IFNAR1 and the ISGs E12AK2, IFI44, ISG15 and Mx1 were measured in 100 pDCs and in PBMCs from HCV-HIV-1 co-infected individuals with minimal to moderate fibrosis (dark green) and with severe fibrosis (light green) using the BioMark. An “ISG score” was defined by the average expression of the four aforementioned ISGs. In pDCs, IFNAR1 expression is not correlated to individual ISG E12AK2 (A) but is negatively correlated with ISG score (B). In contrast, in PBMCs, IFNAR1 expression is positively correlated to E12AK2 (C). Data on IL28B polymorphism (rs12979860) were collected for 8 patients. IFNAR1 expression in pDCs is shown according to IL28B genotype (D).



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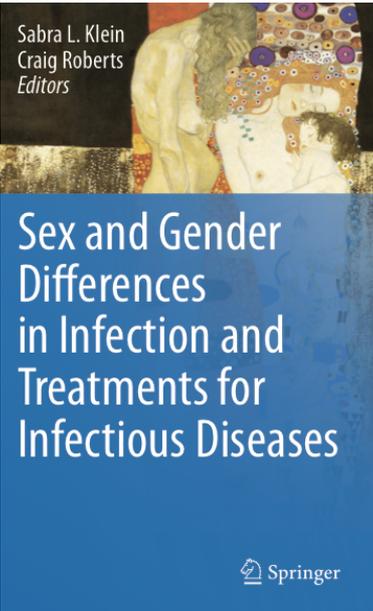
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## **Annexes**





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Craig Roberts  
Editors

## Sex and Gender Differences in Infection and Treatments for Infectious Diseases

 Springer

### Chapter 5 Sex Differences in the Manifestations of HIV-1 Infection

Morgane Griesbeck and Marcus Altfeld

**Abstract** Sex differences have been reported for multiple aspects of human immunodeficiency virus (HIV)-1 infection, including transmission, pathogenesis, morbidity, mortality, and responses to antiretroviral treatment. Epidemiological studies on sex differences in HIV-1 infection are numerous and in some instances controversial. The intrinsic interplay between multilayered socioeconomic factors and biological factors renders drawing definitive conclusions on sex differences in HIV-1 infection challenging. Nevertheless, some findings such as the lower viremia observed in women than in men have been consistently observed in multiple studies. It is also generally acknowledged that women display a greater susceptibility to HIV-1 acquisition. The simian immunodeficiency virus (SIV) model has been critical for understanding the biological characteristics of the female genital tract responsible for this greater susceptibility. Immune activation is another key factor in HIV-1 acquisition and pathogenesis that distinguishes men and women, with women exhibiting higher levels of immune activation. Data to date have pointed toward an important role of sex hormones in mediating these sex differences observed in the manifestation of the HIV-1 disease. This chapter will focus on the discussion of (1) sex differences in HIV-1 acquisition and transmission, (2) sex differences in HIV-1 pathology, and (3) the impact of sex hormones including those exogenously delivered during contraceptive use. Sex differences related to responses to antiretroviral treatment go beyond the scope of this chapter and are reviewed in Chap. 4.



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# High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry

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Fluorescent *in situ* hybridization (FISH) is a method that uses fluorescent probes to detect specific nucleic acid sequences at the single-cell level. Here we describe optimized protocols that exploit a highly sensitive FISH method based on branched DNA technology to detect mRNA and miRNA in human leukocytes. This technique can be multiplexed and combined with fluorescent antibody protein staining to address a variety of questions in heterogeneous cell populations. We demonstrate antigen-specific upregulation of IFN $\gamma$  and IL-2 mRNAs in HIV- and CMV-specific T cells. We show simultaneous detection of cytokine mRNA and corresponding protein in single cells. We apply this method to detect mRNAs for which flow antibodies against the corresponding proteins are poor or are not available. We use this technique to show modulation of a microRNA critical for T-cell function, miR-155. We adapt this assay for simultaneous detection of mRNA and proteins by ImageStream technology.

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Single-cell analysis is a rapidly growing field in biomedical research that yields an unprecedented level of information<sup>1,2</sup>. Flow cytometry has been the gold standard for high-throughput phenotypic and functional characterization of various cell types. While the major advantages of flow cytometry include a simple workflow using fluorescently labelled antibodies for the detection of intracellular or surface markers and high throughput, this technology is limited by availability of highly specific and sensitive reagents for antigens.

Advances in molecular biology have enabled techniques such as microarrays, quantitative PCR and RNA sequencing<sup>3–5</sup>. These technologies are sequence based and not constrained by antibody availability, providing ‘unrestricted’ tools for in-depth analysis of transcriptional signatures that define cell functions in physiologic or disease states. The major drawback of most of these approaches is the generation of transcriptional signatures in bulk populations of cells, which may provide insufficient and/or spurious information on the biologic entity of rare subsets within heterogeneous populations<sup>6</sup>. More recently, new methods such as microfluidic platforms and adaptations of RNAseq have been successfully used for single-cell transcriptional analysis<sup>7,8</sup>. However, for these techniques, single cells must be first live sorted and the laborious methods required to isolate and amplify RNA can result in a significant loss of RNA species<sup>9</sup>.

Fluorescent *in situ* hybridization (FISH) is another method used for the detection of specific nucleic acid sequences at the single-cell level<sup>10</sup>. It is based on fluorescently labelled probes and has been widely used in fluorescence microscopy to detect RNA or DNA sequences in cells adhered to coverslips or tissues<sup>11</sup>. Even though this powerful imaging technique enables detection of down to a single copy of a given nucleic acid sequence<sup>12</sup> and simultaneous analysis of intracellular structures and localization, it has so far been poorly adaptable to flow cytometry. Flow cytometric identification of RNA species would have several major comparative advantages. Whereas microscopy is practically limited to analysis of a few hundred cells, flow cytometry allows high-throughput acquisition of up to millions of cells in a single sample and combination of larger varieties of fluorescent channels; acquisition of 10–15 fluorescent markers are now routinely performed on four- or five-laser cytometers.

Several adaptations of microscopy methods have been attempted in the past to detect FISH signals with flow cytometry<sup>13–15</sup>. However, they were limited to nucleic acid sequences with abundant expression, such as viral RNAs after acute infection<sup>14</sup>. More recently, another study<sup>16</sup> achieved detection of lower levels of RNA and oncogene expression in cell lines, and identified viral genes (that is, human immunodeficiency virus (HIV)) after *in vitro* infection of primary samples. Another limitation of previous experimental approaches is the inability to combine mRNA detection with antibody staining of proteins, a critical tool to define specific cell subsets in mixed populations.

Here we describe a novel flow-FISH method for high-throughput detection of mRNA and microRNA (miRNA). This method is derived from the QuantiGene ViewRNA FISH cell assay (RNA-FISH) that was developed by Affymetrix for microscopy<sup>17</sup>. We show here that new protocols developed in our laboratory, when used in combination with this technique, enable the simultaneous detection of several mRNA molecules in various leukocyte subsets from human blood identified by antibody staining of cell surface markers. We demonstrate sensitive and specific detection of cytokines such as interferon- $\gamma$  (IFN $\gamma$ ) and interleukin (IL)-2 mRNA of HIV- and cytomegalovirus (CMV)-specific CD4 and CD8 T cells. We confirm specificity of cytokine mRNA detection by simultaneous IFN $\gamma$  mRNA-FISH and IFN $\gamma$  protein secretion capture assays.

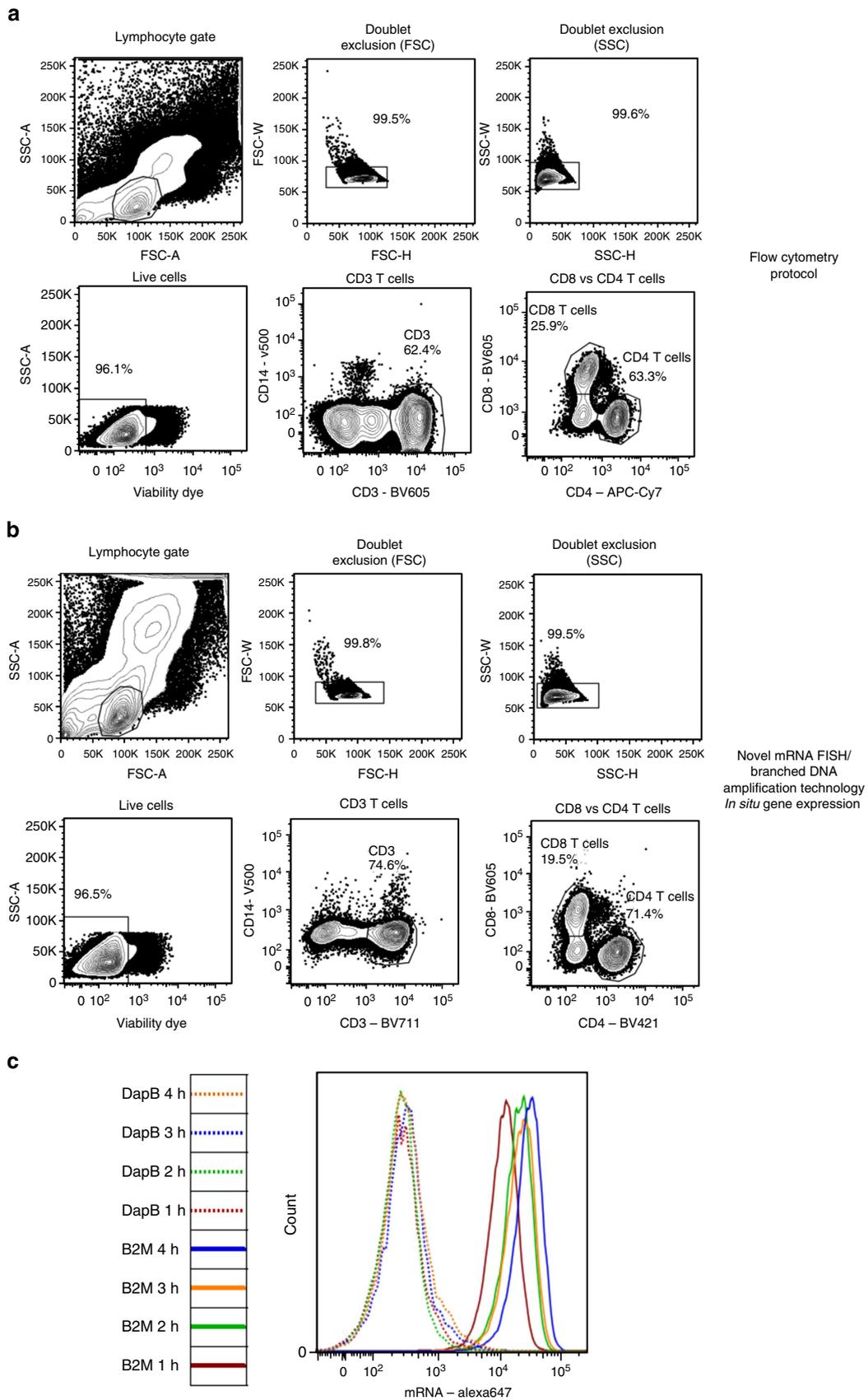
We highlight the ability of this assay to overcome the limitation of antibody availability by excellent detection of IL-21, a cytokine for which intracellular cytokine staining (ICS) with a fluorescent antibody is not sensitive enough to allow adequate quantitation. Furthermore, we use this assay to detect the expression of Indoleamine 2,3-Dioxygenase (IDO), an enzyme for which no flow cytometry antibody was available at the time these experiments were performed. Similarly, we show that this technique can be readily used for the detection of small miRNA molecules that play major regulatory roles in eukaryotic cells without being translated into proteins. Finally, we prove that this approach can be used in combination with ImageStream technology, allowing for high-throughput sub-cellular compartmentalization studies of mRNA and protein co-expression. Taken together, these results show the power and high versatility of this novel technology to address biomolecular mechanisms in heterogeneous cell populations.

## Results

### Detection of mRNA and antibody staining by flow cytometry.

One of the major limitations of typical FISH protocols is the variable requirement in hybridization times and conditions for different probe sets. In contrast, a specific algorithm is used for the QuantiGene ViewRNA method that allows for the design of probes that exhibit high sensitivity and specificity as well as the same hybridization temperature. This provides greater reproducibility between different probes and experimental designs and facilitates assay multiplexing. A prerequisite for the use of this technology in flow cytometry is the availability of hybridization and staining reagents compatible with cells in suspension. Initial testing revealed that cell buoyancy and cell lysis were major issues with the buffers developed for adherent cells and fluorescent microscopy, resulting in massive subsequent cell loss (Supplementary Fig. 1). Even though we were still able to reliably detect 18 s ribosomal RNA with flow cytometry, such a large cell loss, along with the incompatibility between the initial FISH protocol and antibody staining, made this protocol unfavourable for the analysis of primary cells in general and rare subpopulations, such as antigen-specific T cells in particular.

Through a collaborative effort, we therefore developed alternative methods to process non-adherent cells. The cells were fixed with a methanol-based reagent before permeabilization. This new protocol was less harsh to the cells, leading to reduced cell loss and preservation of the main morphologic characteristics as indicated by forward and side scatter (Fig. 1a,b). We next performed head-to-head comparison of antibody staining using a standard surface stain flow cytometry protocol or the new flow-RNA assay. To this purpose, we selected fluorochromes resistant to methanol fixation<sup>18</sup> that stained for surface markers identifying main peripheral blood mononuclear cell (PBMC) subsets. In our hands, Brilliant Violet, Alexa and eFluors dyes perform particularly well. We assessed the respective ability of specific antibody clones to recognize epitopes that withstand the procedures. As previously reported, the potential usability of an antibody depends on both the protein targeted and the specific clone. Although several manufacturers provide useful references as to which reagents are suitable with methanol-based flow protocols, in our experience this use still requires careful assessment by the investigator for each specific mRNA flow-FISH application. Once we had identified appropriate combinations, surface staining for PBMC subsets combined with the Flow-RNA assay gave excellent results, with definition of cell populations very similar to a standard flow cytometry protocol (Fig. 1a,b). Furthermore, the new buffer used is compatible with dead cell viability dyes (Fig. 1b).

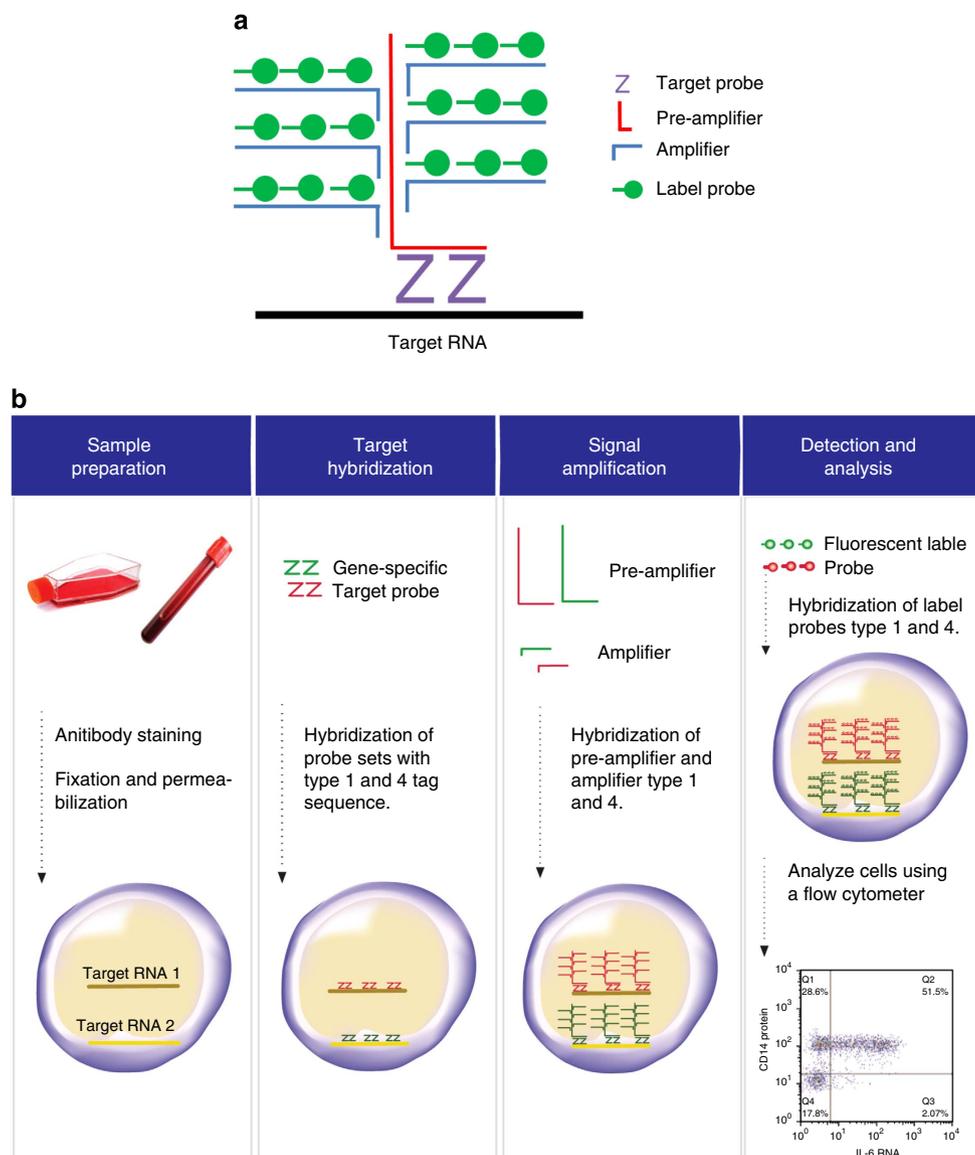


**Figure 1 | Antibody staining and detection of housekeeping genes.** Comparison of physical and phenotypic characteristics of PBMCs stained with a dead cell dye and fluorescent antibodies for CD3, CD4, CD8 and CD14 using either (a) a standard surface stain flow cytometry protocol or (b) the new flow-RNA assay (representative out of five). (c) Kinetic experiment of different hybridization incubation times following staining with either an irrelevant probe for the bacterial gene DapB or the housekeeping gene  $\beta$ 2M (representative example out of three).

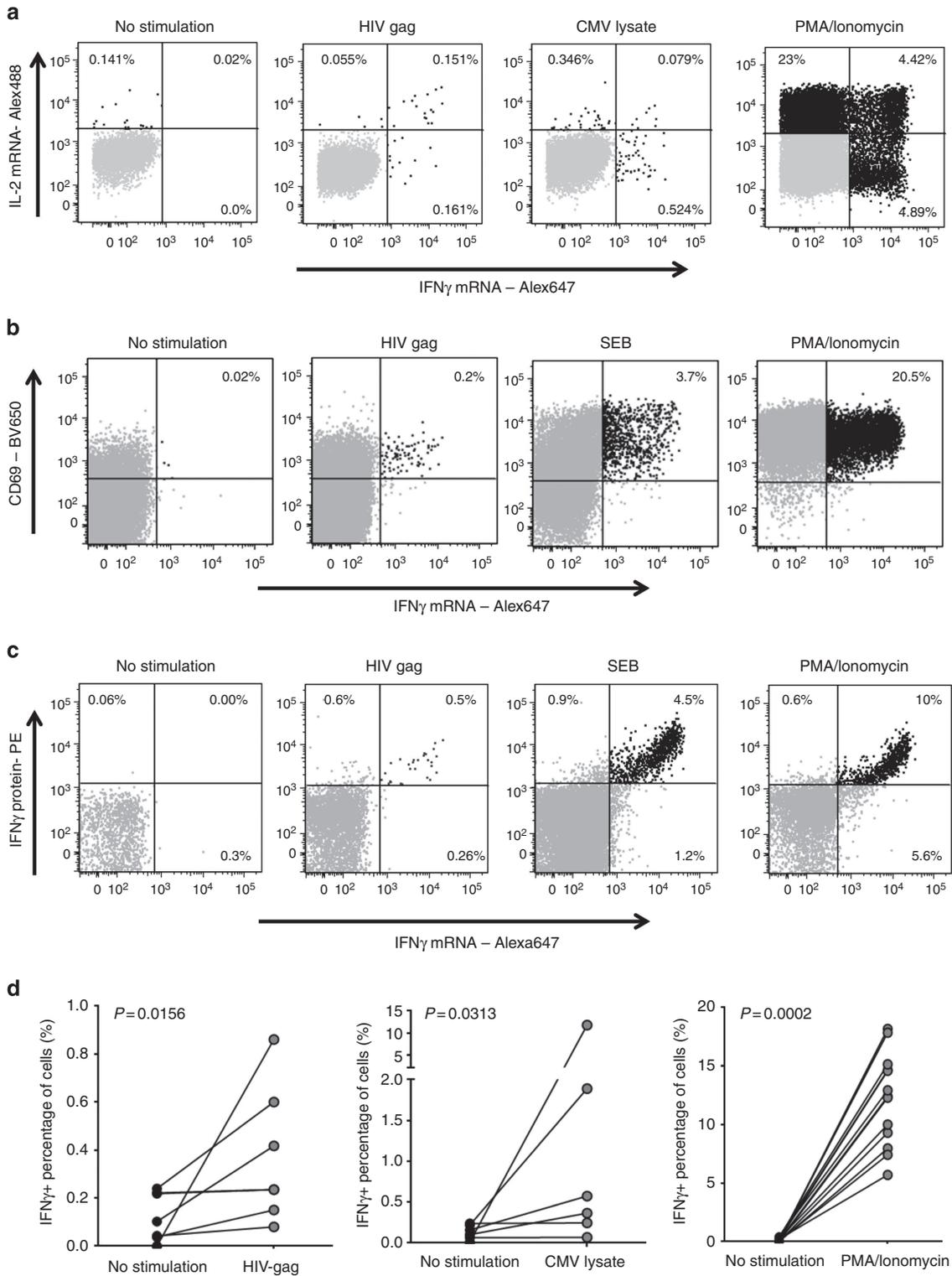
The mRNA probes have optimal hybridization temperature of about 40°C. To identify the hybridization time that confers optimal signal-to-noise ratio for detection, we performed a kinetics experiment using either an irrelevant probe towards DapB (dihydropicolinate reductase of *Bacillus subtilis*) or a probe specific for the housekeeping mRNA  $\beta$ 2-microglobulin ( $\beta$ 2M). A plateau was reached between the 2- and 4-h hybridization times (Fig. 1c), with excellent signal-to-noise ratio. We thus selected the 2-h hybridization step for subsequent experiments. A schematic representation of the workflow can be found in Fig. 2.

The sensitivity of the assay varies depending on the probes used (as different probes have different affinities) and the target sequence of interest (small RNA sequences are targeted by fewer probes resulting in potentially weaker signal). On the basis of our preliminary data shown in Supplementary Fig. 2, we can confidently detect about 10 mRNA copies per cell for the housekeeping gene HMBS (hydroxymethylbilane synthase). It is recommended that users should perform their own validation to identify the sensitivity of the assay based on the target of interest, the cell type and the specific experimental design. Sequences of the probes used in our studies are detailed in Supplementary Table 1.

**Detection of cytokine-producing antigen-specific T cells.** To investigate the ability of this assay to detect antigen-specific T-cell responses after stimulation with cognate antigen, we examined blood samples from HIV-infected subjects. PBMC were stimulated overnight with a HIV-Gag peptide pool, CMV lysate or Phorbol 12-myristate 13-acetate (PMA)/ionomycin, or left unstimulated. We then performed the flow-RNA assay to detect mRNA levels of IL-2 and IFN $\gamma$  in the CD4 and CD8 T-cell subsets identified by the relevant surface markers. Results demonstrate that we can accurately identify cytokine mRNA-producing virus-specific CD4 T cells (Fig. 3a–d) and CD8 T cells (Supplementary Fig. 3). In contrast to regular ICS where addition of Brefeldin and/or Monensin traps cytokines inside the cell for 6 to 12 h, leading to the accumulation of proteins produced over the duration of incubation; here, we detect an accurate ‘snapshot’ of mRNA expression at the specific time when the cells are fixed. Transcription and translation kinetics will also vary. Therefore, percentages of cytokine-producing cells after stimulation can differ between ICS and flow-mRNA assays. In spite of these differences, there was overall a good correlation between results when transcription (mRNA flow-FISH) and



**Figure 2 | Schematic representation of Flow-FISH methodology** (a) Structure of target probe hybridization with the amplifying branched DNA sequences and the label DNA probes. (b) Diagram representing the workflow of the flow-FISH assay.



**Figure 3 | IL-2 and IFN $\gamma$  mRNA detection in antigen-specific CD4 T cells.** (a) IL-2 and IFN $\gamma$  mRNA detection in CD4 T cells from PBMCs of an HIV-infected individual stimulated with a HIV-Gag peptide pool, CMV lysate or PMA/ionomycin for 12 h (one representative experiment out of five). (b) IFN $\gamma$  mRNA detection in combination with CD69 antibody staining from PBMCs of an HIV-infected subject left incubated with medium alone or stimulated with an HIV-Gag peptide pool, SEB or PMA/ionomycin for 12 h. (c) Combined detection of IFN $\gamma$  protein, using a 45 min capture assay, and IFN $\gamma$  mRNA in CD4 T cells from PBMCs of an HIV-infected person incubated with medium alone or stimulated with an HIV-Gag peptide pool, SEB or PMA/ionomycin for 12 h (one representative experiment out of five). (d) Collective data of IFN $\gamma$  mRNA detection after HIV-Gag peptide pool, CMV lysate or PMA/ionomycin stimulation. Statistical analysis was performed using a Wilcoxon matched-pairs test.

translation (ICS) of IFN $\gamma$  and IL-2 were examined in parallel (Supplementary Fig. 4). The absence of cytokine secretion blockade in the flow-FISH approach is a significant advantage

over standard ICS when cell-cell interactions are examined, as many cytokines work in autocrine and paracrine fashion to modulate activation signals.

To prove that the detection of mRNA is specific to activated cells, we utilized two different approaches. We first used an antibody against CD69, a surface molecule upregulated on most T cells on stimulation, in combination with mRNA detection. The large majority of IFN- $\gamma$  mRNA+ cells were also CD69+, indicating specific detection of mRNA in the activated population (Fig. 3b). We next performed the flow-RNA assay in combination with a cytokine capture assay (Miltenyi Biotec, used per manufacturer's instructions) that allows trapping of the molecule of interest by a primary antibody coated on the cell surface, followed by detection with a secondary fluorescent-labelled antibody. The IFN- $\gamma$  capture phase lasts for 45 min and therefore stains cells that have secreted IFN- $\gamma$  within this time frame. Figure 3c shows combined detection of IFN- $\gamma$  protein by IFN- $\gamma$  capture and mRNA production in HIV-specific T cells stimulated with HIV-Gag or T cells stimulated with Staphylococcal enterotoxin B (SEB) or PMA. These data show overall very good consistency; however, after SEB and PMA/ionomycin stimulation we observed a small percentage of cells harbouring IFN $\gamma$  mRNA but on which no IFN- $\gamma$  protein was detected by the capture assay: about 20% of the CD4 T cells expressing IFN $\gamma$  mRNA on SEB stimulation and 35% of the CD4 T cells expressing IFN $\gamma$  mRNA on PMA stimulation were negative by IFN $\gamma$  capture. This difference may be due to both a difference in sensitivity as well as different in kinetics with a delay between transcription and translation. These data show that the flow-RNA assay is sensitive enough to detect antigen-specific T cells and can be used to detect cytokine transcription and translation in the same cells, which can be very useful for studies of functional regulation of immune cells.

#### Sensitive detection of molecules with poor antibody staining.

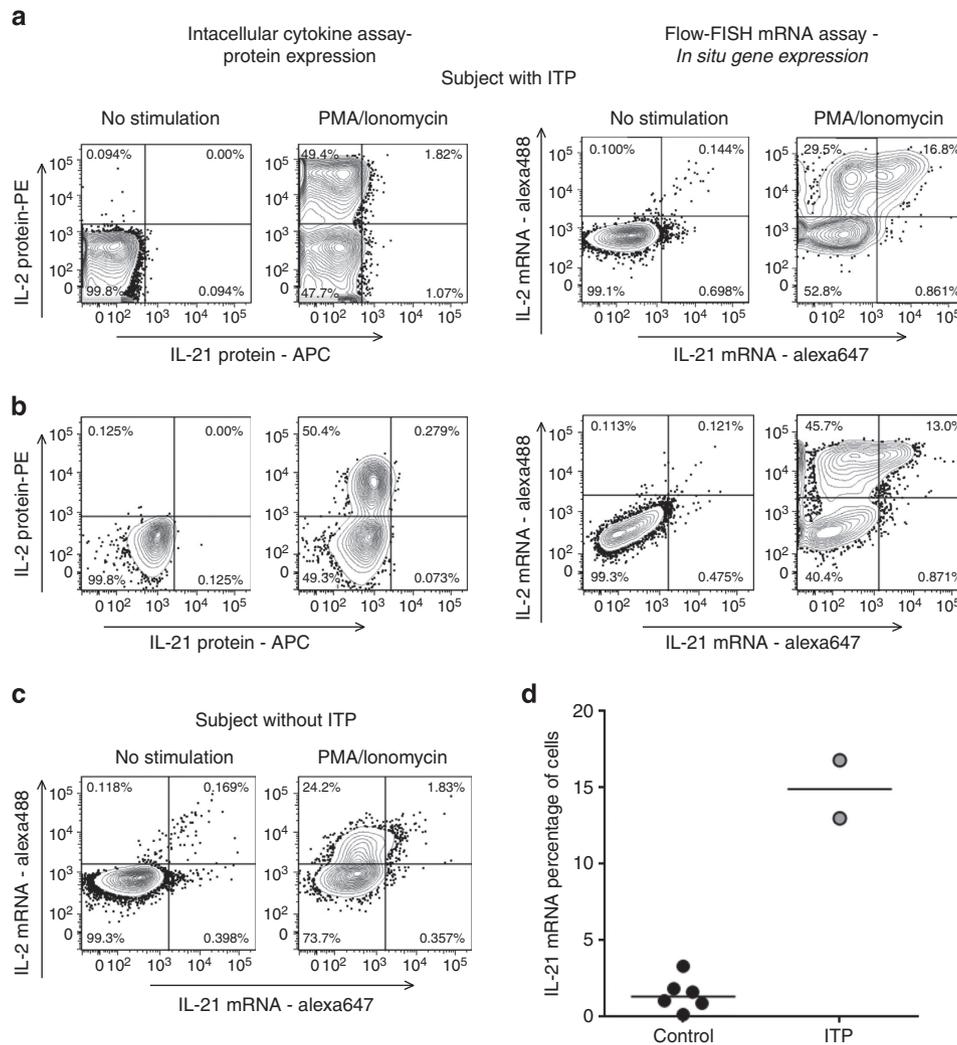
One of the major limitations of antibody-based flow cytometry is that although thousands of suitable antibodies are commercially available, these still do not cover the full panel of molecules of interest present within a cell. Moreover, antibodies have variable sensitivities and often do not perform well at detecting weakly expressed antigens. Flow-RNA offers the potential to stain for a much more diverse selection of molecules. To test this, we first decided to stain for the cytokine IL-21, as the low sensitivity of its detection by ICS has been a hurdle for many investigators. IL-21 is a cytokine critical for CD4 T cell help to B cells and CD8 T cells, but is also implicated in several autoimmune diseases<sup>19</sup>, including idiopathic thrombocytopenic purpura (ITP). ITP is characterized by abnormal production of antibodies that attack platelets. These autoantibodies have been linked to abnormal T follicular helper cells that produce increased amounts of IL-21 (ref. 20). We thus decided to test the ability of the mRNA-FISH assay to detect IL-21 compared with ICS in spleen mononuclear cell samples from two subjects that had undergone splenectomy for ITP refractory to medical treatment and from one individual who had surgery for traumatic splenic rupture. Despite maximal stimulation with PMA/ionomycin, ICS assays on splenocytes detected only small levels of IL-21 protein in all three subjects investigated (Fig. 4), whereas robust production of IL-2 was identified. In contrast to ICS, the mRNA flow-FISH assay was able to detect a much greater percentage of IL-21-secreting cells after PMA/ionomycin stimulation, whereas the fraction of IL-2-secreting CD4 T cells was overall similar with the two techniques. Interestingly, this massive production of IL-21 was present in the two ITP subjects (Fig. 4a,b) but very low in the control sample (Fig. 4c). Extension of these experiments to five additional subjects who underwent splenectomy for indications unrelated to hematologic or autoimmune disorders confirmed low IL-21 mRNA expression compared to individuals with ITP (Fig. 4d). Detection of IL-21 mRNA was restricted to CD4 T cells and not CD8 T cells (Supplementary Fig. 5), consistent with the known role

of CD4 T cells as the main producers of IL-21. We were able to simultaneously use three different probes to detect mRNA levels of IL-2 (Alexa 488), IL-21 (Alexa 647) and IFN- $\gamma$  (Alexa 750) in CD4 T cells from these patients (Supplementary Fig. 6), allowing for detailed investigation of co-expression patterns at the single-cell level.

We next decided to investigate expression of an enzyme for which there was no commercially available antibody for flow cytometry at the time that this experiment was performed, IDO. An antibody detecting intracellular IDO became recently available (eBioscience). IDO is critical for metabolism of tryptophan, an essential amino acid. Catabolism of tryptophan by IDO results in an immunosuppressive environment that has been implicated in the pathogenesis of cancer, autoimmunity and chronic infections<sup>21,22</sup>. Stimulation of PBMCs with the TLR7/8 agonist ligand R848 or with combination of IFN- $\gamma$  and lipopolysaccharide (LPS) for 8 h led to robust upregulation of IDO mRNA in monocytes (Fig. 5a,b). We also found a strong correlation between IDO mRNA expression in CD14+ cells by flow-FISH and the relative mRNA levels as measured by quantitative reverse transcription-PCR (qRT-PCR; Fig. 5c). These data show the unique capacity of the flow-FISH assay to detect mRNA expression of molecules that cannot be detected with antibody-based flow cytometry.

**miRNA detection in primary T cells by flow cytometry.** We next utilized this technique to detect miRNA expression in primary T cells after PMA/ionomycin stimulation. miRNAs are small non-coding RNAs that control gene expression at the post-transcriptional level and play a critical role in regulating cell functions. Here we used flow-FISH to detect two miRNA targets, miR-21 and miR-155 that have been shown to control immune responses<sup>23,24</sup>. Stimulation of CD4 and CD8 T cells for 12 h did not significantly enhance miR-21 expression but led to increased expression of miR-155 (Fig. 6). This is consistent with the literature that showed miR-155 to be one of the few miRNAs upregulated in T cells after stimulation, whereas miR-21 is not significantly enhanced<sup>25</sup>. miRNA expression was measured in activated CD69+ CD4 (Fig. 6d) and CD8 T cells (Fig. 6c) proving the ability of flow-RNA to provide insight into single-cell co-expression of miRNAs in combination with mRNAs molecules and proteins of interest. These data show the unprecedented capabilities of this novel method to also quantify small non-coding miRNA molecules that act as critical regulators of mRNA translation.

**Combined mRNA and protein visualization using ImageStream.** One of the disadvantages of any traditional flow cytometry technique compared with microscopy is that one cannot visualize subcellular localization of molecules. The newly developed ImageStream technology (Amnis) combines the advantages of high-throughput flow stream analyses with microscopy capabilities. Although not reaching the spatial definition of standard fluorescent microscopy, this platform allows much easier acquisition of statistically significant cell numbers in heterogeneous populations. Developing combined detection of multiple mRNAs and proteins with this technology thus has the potential of considerably expanding the versatility of its applications. To assess the applicability of the flow-RNA protocol to the ImageStream platform, we again used a cytokine protein capture assay combined with cytokine mRNA quantitation. We were able to simultaneously detect IFN- $\gamma$  protein and IFN- $\gamma$  mRNA in blood CD4 T cells from a healthy subject (Fig. 7). These data show the potential application of flow-RNA to perform ImageStream experiments for high-throughput co-visualization of mRNA, miRNA and protein at the single-cell level and



**Figure 4 | Detection of IL-21 mRNA in CD4 T cells.** Lymphocytes isolated from a spleen sample of two subjects suffering from ITP (**a,b**) and one subject who underwent surgery for traumatic splenic rupture (**c**). Cells were incubated with culture medium alone or stimulated overnight with PMA/ionomycin, with addition of brefeldin and monensin only for the ICS assay. Following incubation, cells were stained either following a standard ICS protocol or with the new flow-RNA-FISH protocol for dead cell dye, surface lineage markers and either cytokine-specific antibodies or cytokine-specific probe sets. (**d**) Collective data on IL-21 mRNA expression after PMA/ionomycin stimulation in CD4 T cells from six negative control and two ITP subjects.

briefly demonstrate the potential for flow-FISH in co-localization studies. This approach may be applied to investigations of cellular localization in protein–mRNA or miRNA–target mRNA interaction studies.

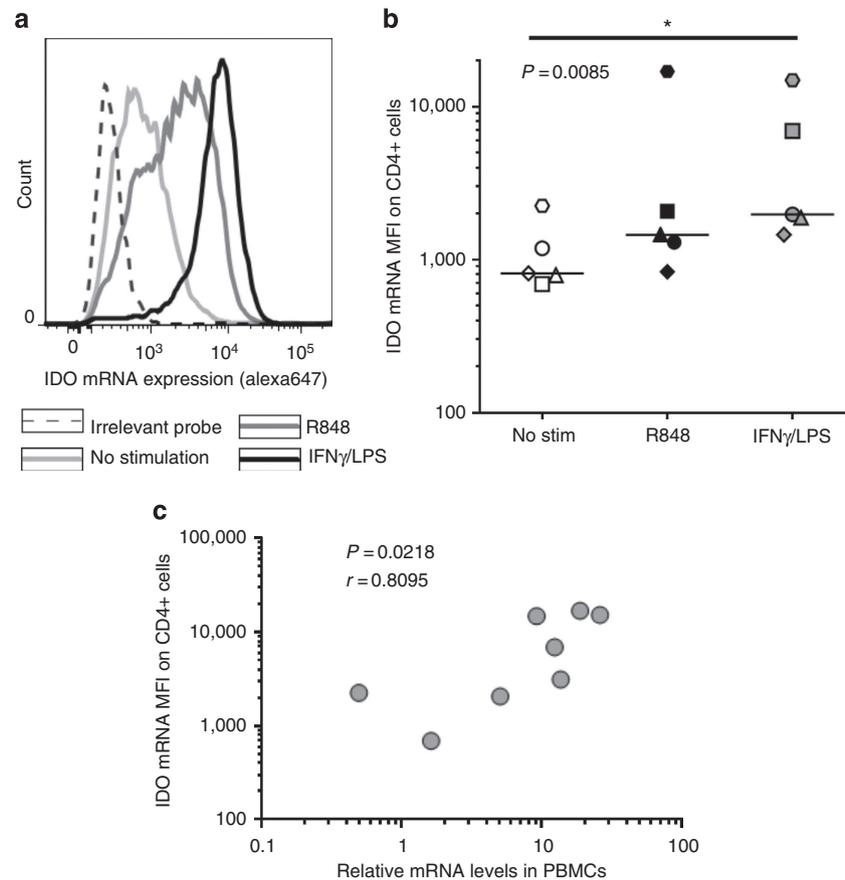
**Discussion**

Fluorescent *in situ* hybridization (FISH) enables the detection of nucleic acid sequences at the single-cell level. Until now, this powerful technology has been largely limited to fluorescent microscopy imaging, thus limiting its use for analysis of heterogeneous cell populations, which require acquisition of large numbers of events. Even though a number of attempts have been made to adapt this method to flow cytometry, they overall showed reduced sensitivity, often relatively poor specificity, time-consuming experimental design and lack of versatility with different nucleic acid targets requiring individualized protocols. Critically, they did not allow simultaneous detection of multiple targets in the same sample, and were hampered by the lack of compatibility between RNA-FISH and antibody staining for cell surface markers. Here we show the application of a novel,

powerful RNA-FISH technology in various optimized flow cytometry protocols that overcome several significant hurdles toward the study of cell function, gene expression and transcriptional regulation in heterogeneous cell samples.

The approach presented here is derived from reagents initially developed for fluorescent microscopy (QuantiGene ViewRNA) that have been successfully used to detect DNA, mRNA and miRNAs sequences<sup>17</sup>. Direct application of the QuantiGene ViewRNA protocol to PBMC samples was accompanied by severe cell loss. Furthermore, addition of protease, which enhances accessibility of probes to their target, resulted in poor antibody staining for cell surface markers, probably because of alteration in antigen conformation.

We therefore made use of a new protocol using a methanol-based permeabilization step. We show here that this procedure is compatible with dead cell dyes and, critically, antibody staining for a variety of cell surface proteins. The depth of data provided is thus similar to standard polychromatic flow cytometry. Reagents currently available allow multiplexing for three different sets of probes for the fluorochromes Alexa 488, 647 and 750. The method is compatible with a wide array of small-molecule dyes



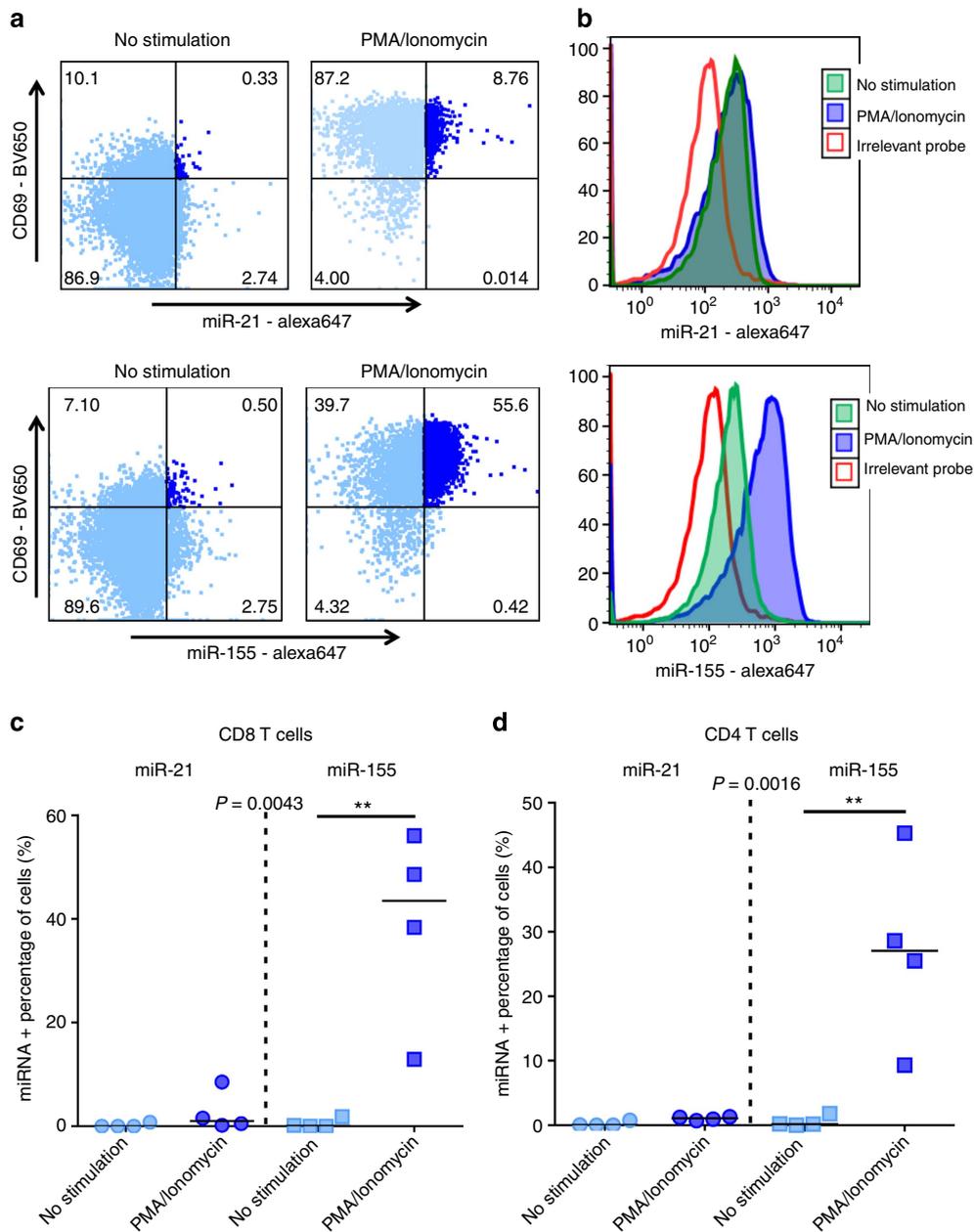
**Figure 5 | Detection of IDO in monocytes.** PBMCs from an HIV-negative subject were stimulated for 8 h with either R848 or IFN $\gamma$  + LPS. IDO mRNA detection in monocytes was achieved after gating on the CD14 positive cell subset within the monocyte gate. **(a)** Representative example and **(b)** collective data on five different subjects. Statistical analysis used Friedman test with a Dunn's post-test comparison. **(c)** Correlation between IDO mRNA mean fluorescent intensity (MFI) in CD14+ cells and IDO relative mRNA expression as measured by qPCR in PBMCs from the same experiment. Statistical analysis used the Spearman rank sum test.

such as Brilliant Violet, Alexa dyes and eFluors, but not with protein-based fluorescent dyes, such as PE, PerCP and APC, and their tandem dye conjugates. Methanol is also known to denature some epitopes, such that some monoclonal antibodies are necessarily incompatible with this method.

After having optimized hybridization times and combination of mRNA probes, surface antibody staining and dead cell dye, we applied the protocol to PMBCs isolated from HIV-infected individuals. We were able to accurately identify CD4 and CD8 T cells and to detect mRNA expression for the cytokines IFN- $\gamma$  and IL-2 by HIV- and CMV-specific T cells after cognate antigen stimulation, with sensitivity comparable to standard ICS for the corresponding cytokine proteins. This mRNA upregulation was exclusively observed within CD69+ cells, confirming that it occurred on activation. To compare transcription and translation levels of the same cytokine, we were next able to combine an IFN- $\gamma$  capture assay that traps secreted proteins on the cell surface with mRNA detection. Besides further confirming the validity of the assay, we believe that this combined assay opens up interesting possibilities to study regulation of cytokine or chemokine production in mixed populations of immune cells. Depending on the question to be addressed, combination with the capture assay can also overcome one current limitation of the mRNA-FISH assay, which is its incompatibility with intracellular staining for proteins. Technically, this limitation can probably be overcome by modification of the fixation and permeabilization procedures, but this will require further investigation.

A major strength of this assay is the potential to detect many mRNAs of interest without being limited by antibody availability. This has great applicability for studies on animal species for which antibody availability for flow cytometry is limited. All FISH probes are derived by an algorithm that enables them to have similar hybridization temperatures and therefore allows combination of different probes and reproducibility between different assays. We were able to detect a combination of three different probes for the cytokines IL-2, IL-21 and IFN- $\gamma$  in the same sample, highlighting the unique multiplexing ability of this method. Besides the large panel of ready-made probes available, custom-designed probes can also be ordered for the gene of interest.

We also assessed the ability of the flow-RNA assay to detect expression of mRNAs for which detection with antibody is either poor or not available. Detection of IL-21 mRNA was clearly superior to identification of IL-21 protein by standard ICS assay. The potential significance of this finding is illustrated by our results on spleen samples from two subjects with ITP, an autoimmune disease in which IL-21 is known to play a role. Whereas ICS revealed little, we found a marked upregulation of IL-21 transcription in CD4+ splenocytes on stimulation in these patients, contrasting with low induction in six subjects with no underlying autoimmune disorder. This technique can thus be used to define altered gene expression patterns in primary cells in the context of human diseases. We could also clearly detect the upregulation of transcription of the immunomodulating enzyme IDO in monocytes after stimulation with TLR agonists, whereas



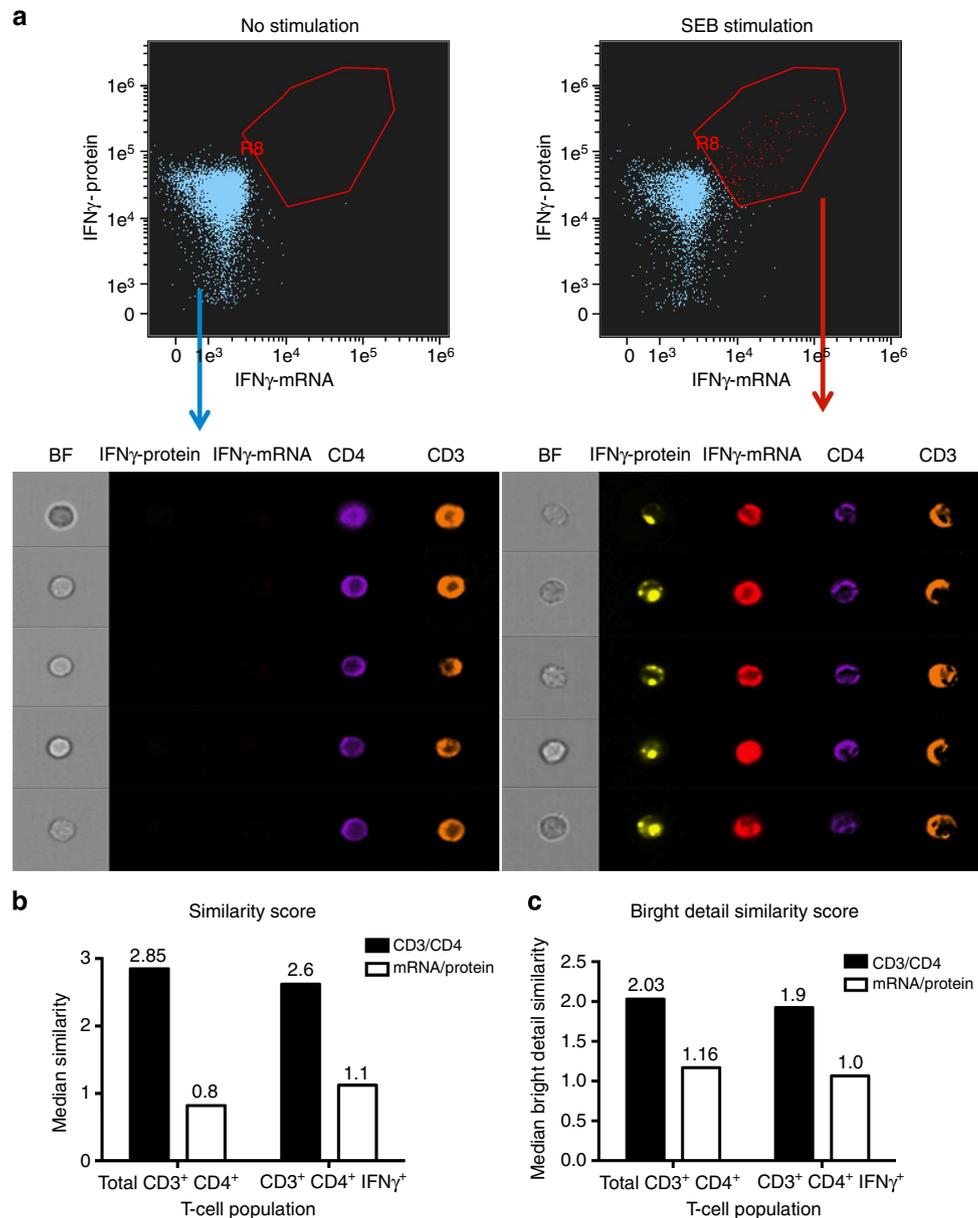
**Figure 6 | miRNA detection in CD4 and CD8 T cells with flow cytometry.** PBMCs from HIV-negative subjects were left unstimulated or were stimulated overnight with PMA/ionomycin. **(a,b)** Representative example of miR-21 and miR-155 expression in CD8 T cells. **(c,d)** Collective data on four subjects for the detection of miR-21 and miR-155 in CD69 + activated CD8 and CD4 T cells. Statistical analysis used Friedman test with a Dunn’s post-test comparison.

no antibody was commercially available to detect IDO by flow cytometry at the time of these experiments.

Recently, it has become increasingly clear that non-coding RNAs, including miRNAs and long non-coding RNAs, play broad and critical regulatory roles in cell regulation, including immune responses. We thus also investigated the ability of mRNA flow-FISH to detect such nucleic acids sequences for which there is no protein translation. miRNAs control gene expression at the post-transcriptional level by translational repression or enhanced target degradation. In spite of the short sequence of miRNAs (around 22 nucleotides), we successfully detected miR-155 modulation in CD4 and CD8 T cells after stimulation. The implication of miRNAs in many diseases makes them attractive targets for immunotherapeutic interventions and biomarker development for cancer and autoimmune diseases<sup>26,27</sup>.

Our proof-of-principle experiments indicate the unprecedented capabilities of this assay to simultaneously detect expression of miRNA, mRNA and protein at the single-cell level, providing a tool for in-depth analysis of miRNA function in combination with their translational and expressional effects in both normal and pathologic conditions.

As flow cytometry does not show localization of protein expression within cells, we finally explored the potential of the flow-RNA assay to be used with ImageStream technology. We successfully detected IFN- $\gamma$  mRNA and protein levels on CD4 T cells after SEB stimulation, with excellent signal/noise ratios for the different parameters measured. Thus, this assay can be used with this novel instrument combining some of the advantages of both polychromatic flow cytometry and fluorescent microscopy, including compartmentalization studies.



**Figure 7 | Combined detection of IFN $\gamma$  protein and mRNA with ImageStream.** (a) PBMCs from an HIV-negative subject were left unstimulated or stimulated with SEB for 12 h. At the end of this incubation time, a 45-min capture assay was performed for detection of IFN $\gamma$ -protein, followed by IFN $\gamma$ -mRNA staining with the flow-FISH assay. CD4 T cells were then acquired on an ImageStream instrument. Images illustrate expression of IFN- $\gamma$  protein, IFN- $\gamma$  mRNA, CD4 and CD3 in the lymphocyte gate in unstimulated cells (left panels) and in stimulated cells (right panels). (b,c) Co-localization analysis for CD3/CD4 in black and IFN $\gamma$  protein/mRNA in white using two different analytical tools (b) Similarity score (the log-transformed Pearson's correlation coefficient, measuring the spatial linear correlation between two total stains within a cell mask) and (c) Bright detail similarity score (the log-transformed Pearson's correlation coefficient, measuring the spatial linear correlation between the only the bright regions of two stains within a cell mask). We found a low correlation between secreted IFN $\gamma$  protein and internal mRNA using both measures (median score  $\sim$ 1). In comparison, surface stained CD3 and CD4 were well correlated (median score  $\sim$ 2).

In conclusion, this novel mRNA/miRNA flow-FISH assay presents major advantages over previous technologies including: an algorithm-based controlled design of probes that allows reproducibility and combination of various probes with similar hybridization conditions; the straightforward combination with fluorescent antibody staining that allows simultaneous phenotyping of heterogeneous cell populations; the design of probes for molecules for which no antibody is available; the user-friendly protocol that has many similarities with standard antibody staining procedures for regular flow cytometry; and the processing and data acquisition with standard laboratory equipment

and cytometers. Our data illustrate the versatility of this technique and its broad applicability for analysis of normal cell functions and disease states.

## Methods

**Human subjects.** Peripheral blood was obtained from HIV-infected and uninfected individuals at the Massachusetts General Hospital (MGH), Boston. The study was approved by the MGH Institutional Review Board and written informed consent was obtained from all study participants before enrolment in the study. PBMCs were isolated by Ficoll density centrifugation. Frozen PBMCs were thawed and were cultured in RPMI 1640 medium (Invitrogen) supplemented with HEPES, penicillin, streptomycin and 10% human AB serum (Gemini Bioproducts) (R10 medium).

Non-identifiable excess spleen tissue was obtained at Massachusetts General Hospital under approval by the Partners Human Resource Committee. These surgeries were performed for medical or surgical indications not related to hematologic or autoimmune diseases, with the exception of two patients who underwent splenectomy for refractory ITP, which is currently an uncommon indication for such an intervention. Spleens were dissected into small fragments and subsequently mechanically disrupted to single-cell suspension using a 70- $\mu\text{m}$  cell strainer and a syringe plunger. Following washing in R10 medium containing 2 mM EDTA, cells were strained once more and erythrocytes were lysed using RBC lysis buffer (Qiagen). Cells were immediately cryopreserved for subsequent use.

**Cell stimulation.** Cells were resuspended in 500  $\mu\text{l}$  R10 medium before being stimulated with an HIV-Gag peptide pool (2  $\mu\text{g ml}^{-1}$  per peptide), CMV lysate (1  $\mu\text{g ml}^{-1}$ ), SEB (1  $\mu\text{g ml}^{-1}$ ), PMA/ionomycin (1  $\times$ , Cell Stimulation Cocktail, eBioscience), TLR7/8 agonist R848 (10 ng  $\text{ml}^{-1}$ ) or recombinant IFN $\gamma$  (100 ng  $\text{ml}^{-1}$ ) + LPS (10 ng  $\text{ml}^{-1}$ ).

**Flow-FISH method.** PBMCs were stained with viability dye (Invitrogen LIVE/DEAD) for 30 min at room temperature. Surface antibodies were then added to stain for cell populations of interest (BV711 CD3 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), BV421 CD4 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4), V500 CD8 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: SK1) and BV605 CD14 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: M5E2)). Assay was performed as per manufacturer's instructions (Affymetrix, Inc., <http://media.ebioscience.com/data/pdf/literature/quantigene-flowrna-user-manual.pdf>). In brief, PBMCs were fixed with a 4% paraformaldehyde solution for 1 h at room temperature. Cells were then permeabilized with ice-cold methanol, followed by further fixation with a 4% paraformaldehyde solution for 30 min at room temperature. All buffers used in the following steps were included in the manufacturer's kit. To detect mRNA, probes comprised of ~20 oligonucleotide base pairs against the mRNA of interest were used. Currently, there are three different types of probes with unique tag sequences, named type 1, type 4 and type 6, respectively. Each tag sequence allows the hybridization of specific complementary branched DNA nanostructure with different excitation/emission spectra: type 1–650/668, type 4–495/519 and type 6–749/775. Probes were diluted 1:20 in target probe diluent, added to the cell suspension and allowed to hybridize to the target RNA for 2 h at 40  $^{\circ}\text{C}$ . Excess probe was removed by adding wash buffer followed by centrifugation at 800g for 5 min. Signal amplification was achieved by performing sequential hybridization with DNA branches (that is, Pre-Amplifier and Amplifier). The first DNA branch in the PreAmp Mix was added at a 1:1 ratio and was allowed to hybridize for 1.5 h at 40  $^{\circ}\text{C}$ . Samples were washed with wash buffer, and then the second DNA branch in the Amp Mix was added at a 1:1 ratio and hybridized for 1.5 h at 40  $^{\circ}\text{C}$ . Samples were washed with wash buffer before addition of the label probes. For signal detection, fluorochrome-conjugated label probes were diluted 1:100 in label probe diluent, added to the samples and allowed to hybridize for 1 h at 40  $^{\circ}\text{C}$ . Finally, PMBCs were washed with wash buffer, transferred to the storage buffer and subsequently analysed on a LSR Fortessa, using FACS DIVA software version 7 (BD Biosciences, San Jose CA). Flow cytometric data were analysed with FlowJo Version 7.5.5 (TreeStar). As negative controls, we used probes that target irrelevant sequences for dihydrodipicolinate reductase/dapB gene of the bacterium *Bacillus subtilis* that should not be encountered in human samples. For the experiments where alterations of mRNA levels were investigated after stimulation, the gates were set according to the unstimulated control.

**Optimization of target probe hybridization time.** To determine the optimal hybridization time in terms of the signal-to-noise ratio, we varied the target probe hybridization times from 1 to 4 h. Both a negative control probe, dapB (*Bacillus subtilis*), and a probe for housekeeping gene  $\beta$ 2-microglobulin (type 1 probe) were used.

**mRNA detection in activated CD4 T cells.** PBMCs were either left unstimulated or were stimulated with HIV-Gag, SEB or PMA/ionomycin overnight (16 h). The next day, cells were stained with viability dye and a panel of surface antibodies ((CD3-BV711 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), CD4-BV421 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4), CD8-BV605 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: RPA-T8), CD14-V500 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: M5E2) and CD69-BV650 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: FN50)). The flow-FISH protocol was followed as outlined above, and IFN $\gamma$  mRNA type 4 probes were detected in activated CD4 T cells (CD4 + /CD69 +) on the LSR Fortessa (Fig. 2b).

For detection of IL-21 CD4 T cells from spleen samples of subjects with ITP, we stimulated cells for 12 h with PMA/ionomycin. The flow-FISH protocol was followed as outlined above, and IL-2 (type 4 probes), IL-21 (type 1 probes) and IFN $\gamma$  (type 6 probes) mRNA on CD4 and CD8 T cells were measured on a BD Fortessa (Fig. 3). We also performed ICS from the same samples to compare IL-21 detection between ICS and the new flow-FISH protocol. In brief, cells were stimulated with PMA/ionomycin for 12 h in the presence of Monensin and Brefeldin A (5  $\mu\text{g ml}^{-1}$ ). Subsequently, cells were stained with blue viability dye (Invitrogen Life Technologies) and fluorescent antibodies against CD3 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), CD4 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4), CD8

(5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: SK1), CD14 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: M5E2) and CD19 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: HIB19). Intracellular staining for IFN $\gamma$ , IL-2 and IL-21 was performed using BD Cytfix/Cytoperm Fixation/Permeabilization Solution according to the manufacturer's instructions. Cells were then analysed on the LSR Fortessa.

**Simultaneous detection of cytokine mRNA and protein.** To determine whether the mRNA we were detecting correlated with the cells producing the protein, PBMCs were stimulated overnight with either HIV-Gag peptide pools, SEB or PMA/ionomycin. Cells were then labelled with IFN $\gamma$  capture reagent using the Miltenyi IFN $\gamma$  capture assay kit and protocol. Cells were allowed to secrete cytokines for 45 min before commencing staining. Cells were stained with viability dye and surface antibodies (CD3-BV711 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), CD4-BV421 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4), CD8-V500 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: SK1) and CD69-BV650 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: FN50)), and the flow-FISH was followed. After completing washes outlined at the end of the protocol, cells were labelled with anti-IFN $\gamma$  PE detection reagent following the Miltenyi protocol. The PE detection reagent was added after the flow-FISH protocol was completed because, if added before the fixation and permeabilization steps, the methanol would have degraded the protein dye and no IFN $\gamma$  protein signal would have been observed. Cells were then analysed on the LSR Fortessa.

**IDO mRNA detection with Flow-FISH and qRT-PCR.** PBMCs from HIV-negative subjects were stimulated with R848 or IFN $\gamma$  + LPS for 8 h. After 8 h, the cells were split into two, and half of them were processed with the flow-FISH assay for detection of IDO mRNA (type 4 probe) on CD14 cells (CD3-BV711 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), CD19-V500 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: HIB19) and CD14-BV605 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: M5E2)) and the other half was lysed in RLT buffer containing 1% beta-mercaptoethanol followed by mRNA extraction with the Qiagen mRNA isolation kit. qRT-PCR for IDO and GAPDH housekeeping gene was performed using Taqman probes from ABI according to the manufacturer's instructions.

**Detection of miRNA with flow-FISH.** PBMCs from HIV-negative subjects were either left unstimulated or were stimulated for 12 h with PMA/ionomycin. Flow-FISH protocol was followed as described above. Probes specific for miR-21 and miR-155 (type 1) were used. Cells were stained with surface antibodies (CD3-BV711 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3), CD4-BV421 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4), CD8-V500 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: SK1) and CD69-BV650 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: FN50)) and miRNA probes were detected in activated CD4 T cells (CD4 + /CD69 +) on a LSR Fortessa.

**Imaging flow cytometry to visualize mRNA and protein.** To visualize simultaneous detection of IFN $\gamma$  mRNA and protein PBMCs were left unstimulated or were stimulated with SEB for 12 h. Flow-FISH in combination with the IFN $\gamma$  capture assay were performed as described above. For each sample, 30,000 cells were collected on a five-laser two-camera ImageStream<sup>Mark II</sup> imaging flow cytometer (Amnis, Seattle, WA). This instrument combines the immunofluorescent sensitivity and capacity of a traditional flow cytometer with bright-field high-resolution microscopy and image analysis. Since the ImageStream has an alternate excitation capacity than the LSR Fortessa, alternate antibody fluorochrome conjugates were necessary to achieve optimal detection. For this reason, we used CD8-BV785 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: RPA-T8), CD3-BV605 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT3) and CD4-BV421 (5  $\mu\text{l}$  per 100  $\mu\text{l}$  of sample, clone: OKT4). Images were captured with INSPIRE acquisition software (Amnis v.6.0.154.0) utilizing  $\times 40$  magnification.

Post-acquisition data analysis was performed using IDEAS software (Amnis v.6.0.333.0). Single, focused cells were gated using Gradient RMS (focus), then Area versus Aspect Ratio in the Bright-field (BF) channel. Next, CD3 + events were gated based on Intensity (versus BF area). From the CD3 + population, a bivariate intensity plot of CD4 versus CD8 was generated to gate the CD8 + population.

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## Author contributions

F.P. and D.E.K. were responsible for the overall design and conduct of the experiments; F.P., C.L., Q.N., B.N., S.R., D.G.K. and D.E.K. provided intellectual input and contributed to the experimental design; F.P., M.G.H., M.G., H.L.E., M.H., M.L., S.M.M., D.Z.S., S.M. and D.E.K. performed the experiments; A.E.B. analysed the data; F.P. and D.E.K. wrote the paper; and D.E.K. provided supervision.

## Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

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# Higher Expression of Several Interferon-Stimulated Genes in HIV-1-Infected Females After Adjusting for the Level of Viral Replication

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**Background.** Clinical studies have shown faster disease progression and stronger immune activation in human immunodeficiency virus (HIV)-1-infected females when compared with males for the same level of HIV-1 replication. Here we determine whether the elevated levels of HIV-1-induced interferon-alpha (IFN- $\alpha$ ) production observed in females are associated with higher interferon-stimulated gene (ISG) expression levels in T cells, hence suggesting type-I IFN as a mechanism for the higher HIV-1-associated immune activation observed.

**Methods.** T-cell and dendritic cell populations were isolated from treatment-naive chronically HIV-1-infected individuals enrolled in the Adult Clinical Trials Group 384 by fluorescence-activated cell sorting. The expression of 98 genes involved in Toll-like receptor and type I IFN signaling pathways were quantified using Nanostring technology.

**Results.** Several ISGs were significantly correlated with HIV-1 viral load and/or CD4<sup>+</sup> T-cell count. Higher expression levels of a subset of these ISGs were observed in cells derived from females as compared to males after adjusting for viral load and were correlated to higher levels of T-cell activation.

**Conclusion.** These data show that higher IFN- $\alpha$  production is associated with higher ex vivo expression of several ISGs in females. This might contribute to higher levels of immune activation and the observed faster HIV-1 disease progression in females for a given level of viral replication.

**Keywords.** HIV-1; innate immunity; type I Interferon; Toll-like receptors; T cells; sex differences; immune activation; dendritic cells; pathogenesis.

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Presented in part: Gender differences in the TLR-mediated IFN- $\alpha$  responses are associated with higher expression of interferon stimulated genes in infected women (J.J.C., R.J.L., M.W., E.S.C., E.H.D., G.K.R., R.J.B., and M.A.). Keystone Symposia 2011, Whistler, Canada, 22 March 2011. Poster 136.

Presented in part: Sex differences in the TLR-mediated IFN-alpha responses are associated with higher expression of interferon stimulated genes in infected women (J.J.C., M.W., R.J.L., E.S.C., E.H.C., E.H.D., M.G., R.J.B., and M.A.). AIDS Vaccine 2011 Conference, Bangkok, Thailand, 13 September 2011. Poster P09.01.

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Differences in the clinical manifestations of human immunodeficiency virus (HIV)-1 infection between the sexes have been described, including a lower average viral replication set point in females as compared to males in the early phase of HIV-1 infection [1–3]. Despite this initially lower viral set point, faster disease progression and higher levels of immune activation are subsequently observed in chronic HIV-1 infection in females as compared to males after controlling for viral load [4–7]. The precise mechanisms underlying these sex differences in the manifestations of HIV-1 disease are not well understood. However, previous in vitro studies have shown that plasmacytoid dendritic cells (pDCs) from females produced significantly more interferon-alpha (IFN- $\alpha$ ) in response to HIV-1 and HIV-1-derived Toll-like receptor (TLR)7 ligands than

pDCs from males [6]. HIV-1 can directly induce production of IFN- $\alpha$  by pDCs through the recognition of HIV-1 ssRNA via TLR7 [8, 9] and subvert internal negative regulatory pathways, leading to persistent IFN- $\alpha$  production [6, 10, 11]. However, the in vivo consequences of these observed sex differences in IFN- $\alpha$  production by pDCs on T-cell activation still need to be determined.

Utilizing stored samples from treatment-naive chronically HIV-1-infected individuals with known T-cell activation levels [6], the expression of 98 genes involved in the TLR and type I IFN signaling pathways were examined in populations of sorted T cells and DCs. Expression levels of interferon-stimulated genes (ISGs) were associated with HIV-1 viral loads, CD4<sup>+</sup> T-cell counts, and markers of T-cell activation. Additionally, the expression of a subset of ISGs was also significantly higher in females as compared to males after controlling for HIV-1 viral load. These findings add to earlier work showing that the sex-dependent differences in IFN- $\alpha$  production [6, 12] are associated with sex-dependent differences in expression of some ISGs. The persistent higher expression of IFN- $\alpha$  and ISGs in females may explain, in part, the higher immune activation and subsequent faster disease progression observed in chronically infected females as compared to males for the same level of viral replication [4–6].

## METHODS

### Ethics Statement

All study subjects gave informed consent for the Adult Clinical Trials Group (ACTG) 384 study and for storage of peripheral blood mononuclear cells (PBMCs) for future studies. The Massachusetts General Hospital Institutional Review Board approval was obtained for secondary use of samples.

### Characteristics of Study Subjects

Cryopreserved PBMCs from 38 treatment-naive HIV-1-infected subjects (19 females and 19 males) enrolled in the ACTG 384 study were examined. These patients were selected from a cohort in whom sex-based differences in T-cell activation levels after adjusting for HIV-1 viral load had previously been observed [6]. The patients were restricted to age <55 years and, via frequency-matching between the males and females, to have similar HIV-1 viral loads using the following categories: <10 000, 10 001–50 000, 50 001–100 000, and >100 000 copies/mL. Individuals with hepatitis C virus infection were excluded from the study. There were no significant differences between males and females in terms of HIV-1 viral load (median of  $1.0 \times 10^4$  and  $4.6 \times 10^4$  copies/mL, respectively) and CD4<sup>+</sup> T-cell count (median of 360 and 319 cells/ $\mu$ L, respectively).

**Table 1. Host Genes Examined**

ADAR	CD83	IFNAR2	LGALS3	TFRC
ANXA11	CD86	IFNB1	MDA-5	TLR1
APOBEC3F	CD8A	IL15	MKI67	TLR10
APOBEC3G	CLEC4C	IL1B	MX1	TLR2
APOBEC3H	CXCL10	IL1R1	MX2	TLR3
ATF3	CXCR4	IL1R2	MYD88	TLR4
B2M	Dectin1-CLEC7A	IL-32	NFKB1	TLR5
BST2	EIF2AK2	INFa2	NFKB2	TLR6
CASP1	FOXP3	MAVS	NMI	TLR7
CASP3	GAPDH	IRAK4	OAS1	TLR8
CASP4	HLA-DRA	IRF1	OAS2	TLR9
CCL4	IDO1	IRF2	OAS3	TNF
CCL5	IFI16	IRF3	PIK3	FasR
CCR5	IFIT1	IRF7	PPP1CA	TNFSF10
CCR7	IFIT3	IRF9	RIG-1	TRAF3
CD38	IFNA4	ISG15	SOCS1	TRAF6
CD4	IFNA5	ITGAX	STAT1	TRIM5
CD40	IFNA6	JAK1	STAT2	TYK2
CD69	IFNA8	JAK2	TAP1	
CD80	IFNAR1	LAG3	TBP	

### Measurement of Gene Expression Profile

Cryopreserved PBMCs were thawed and surface stained on ice for identification of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD11c<sup>+</sup> DCs. The cells were then sorted by fluorescence-activated cell sorting using the BD ARIA (BD Biosciences). Cells were immediately lysed in RNeasy Lysis Buffer (RTL) buffer (Qiagen) containing 1%  $\beta$ -mercaptoethanol (Sigma) for mRNA extraction. Probes specific for 98 genes in the TLR pathway, IFN- $\alpha$  pathway, and ISGs were designed (Table 1), manufactured by Nanostring Technologies, and analyzed using the Nanostring nCounter analysis system as previously described [13]. Briefly, 2000–10 000 cells collected in RLT lysis buffer were mixed with the custom-designed probeset and incubated overnight at 65°C. The samples were then loaded onto the Nanostring prep station to remove unbound probes and align the bound probes for reading using the Nanostring digital analyzer. Each sample was spiked with 6 internal positive controls and 8 internal negative controls. The probeset contained 3 housekeeping genes, which cover a range of gene expression levels, and these were used to control for total RNA input in the Nanostring analysis.

### Qualitative Real-Time Polymerase Chain Reaction and RNA Extraction

A small fraction of the cells resuspended in RLT lysis buffer was kept for qualitative real-time polymerase chain reaction (qRT-PCR) analysis. RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocol. qRT-PCR analysis was performed using a QuantiFast SyBR Green RT-PCR kit (Qiagen) as per the manufacturer's protocol, and cDNA amplification was detected using the LightCycler 480

system (Roche). The expression of 5 genes and GAPDH were quantified by qRT-PCR and significantly correlated with the expression detected by Nanostring Technologies (Supplementary Figure 1A). The primers used for qRT-PCR are MX1 (sense GCCACAAGGCACCTAAGTC and antisense ATTCTCCAAA CCTGCGCTCT), CCR5 (sense TTCCACCCGGGAGAGTTT and antisense ATCCAGTGAGAAAAGCCCGT), CXCR4 (sense GGAGGGGATCAGTATATACTTCAG and antisense CTT GTCCGTCATGCTTCTCA), ISG15 (sense GAGAGGCAGCGA ACTCATCT and antisense CTTGAGCTCTGACACCGACA), and IRF7 (sense CTGTTCGGAGAGTGGCTCCT and antisense AGCCCAGGCCTGAAGAT). Three of the genes examined (ISG15, CCR5, and MX1) were found to differ significantly between males and females after adjusting for HIV-1 viral load in respective cell populations using both qRT-PCR and Nanostring methods. Although the results using qRT-PCR and Nanostring technologies correlated, the *R* value was not high, most likely due to differences between the technologies employed by these two methods. Nanostring does not use the same targeting mechanisms as PCR and, as such, the primers used in the qRT-PCR method may not recognize the same targets that are detected using Nanostring technology. Furthermore, qRT-PCR required reverse transcription and amplification of the target sequence for detection, while the Nanostring technology directly detected the mRNA in the sample. Consequently, the qRT-PCR method has the potential to introduce bias.

#### Measurement of Ex Vivo T-cell Activation in HIV-1-Infected Individuals

CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation in freshly isolated PBMCs was measured using flow cytometry according to established ACTG protocols [6]. Activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were defined as the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells that expressed CD38 and HLA-DR.

#### Statistical Analysis

The raw Nanostring gene expression data were normalized using the internal controls. The arithmetic mean plus 3 standard deviations (SDs) of the internal negative controls in each sample was subtracted from that sample's gene expression readout to eliminate any nonspecific mRNA detection. The geometric mean of the 6 internal positive controls was used to normalize the data in order to minimize any assay-to-assay variations. When calculated for all samples, the mean plus SD of the negative controls was  $1.033 \pm 1.077$  and the geometric mean plus SD of the positive controls was  $32\,684.21 \pm 4528.32$ . The gene expressions were then normalized using the geometric mean of 3 housekeeping genes (GAPDH, PPP1CA, and TFRC). Correlations between the relative mRNA expression and log<sub>10</sub> HIV-1 viral load, CD4<sup>+</sup> T-cell count, and age were measured using Pearson test, and multiple comparison was controlled using false discovery rate (FDR) as described by

Benjamini Hochberg methodology [14]. The *P* values pre-FDR adjustment are included in the Supplementary tables. Wilcoxon rank test was used to determine sex-specific differences, and analysis of covariance was used to determine sex-specific differences after adjusting for log<sub>10</sub> HIV-1 viral load. The percentage of genes with expression levels that significantly correlated with log<sub>10</sub> HIV-1 viral load and/or CD4<sup>+</sup> T-cell count was compared with the 3 cell subsets using Fisher exact test. Following statistical analysis, the correlation results were imported into Ingenuity Pathway Analysis for generation of IFN- $\alpha$  signaling pathways (Ingenuity Systems, Redwood City, CA).

## RESULTS

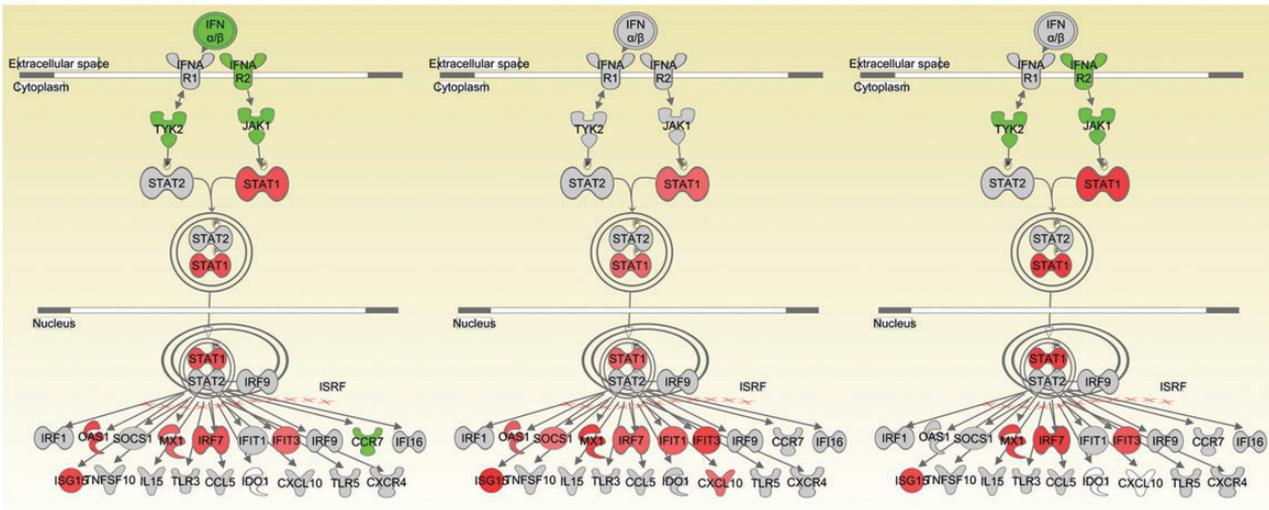
### ISG Expression Positively Correlated With HIV-1 Viral Load and Negatively Correlated With CD4<sup>+</sup> T-Cell Counts

The expression levels of a list of predefined genes involved in the TLR and type I IFN pathways (*n* = 98; see Table 1) were quantified directly ex vivo using flow cytometry-sorted cells (CD11c<sup>+</sup> monocytes and DCs, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells; >99% purity for all subsets) from treatment-naive chronically HIV-1-infected females (*n* = 19) and males (*n* = 19) enrolled in the ACTG 384 study. Sorted cell subsets were used instead of total PBMCs as it has been previously demonstrated that PBMCs may not accurately reflect gene expression profiles of specific cell subpopulations [15, 16]. Using the recently developed Nanostring technology, direct quantification of gene expression without prior amplification of RNA was performed [13]. A significant number of the genes examined were found to be associated with HIV-1 viral load after expression levels were adjusted for FDR using the Benjamini Hochberg methodology [14] (Supplementary Table 1). These included ISGs such as ISG15, MX1, IFIT3, IRF7, and OAS3, for which the expression levels were positively correlated to HIV-1 viral load in all 3 sorted cell populations examined (Figure 1A). As HIV-1 viral loads and CD4<sup>+</sup> T-cell counts were inversely correlated with each other (*r* = -0.43, *P* = .012), the expression levels of several genes were also associated with CD4<sup>+</sup> T-cell counts after adjusting for FDR (Figure 1B, Supplementary Table 2). Expression levels of several of these genes remained significantly negatively correlated to CD4<sup>+</sup> T-cell count after adjusting for HIV-1 viral load and FDR, including ISGs such as MX1, IFIT3, IRF7, OAS2, ISG15, and IFIT1 (Supplementary Table 2). Taken together, this study identified a number of ISGs with expression levels that were significantly associated with the level of viral replication or CD4<sup>+</sup> T-cell counts in treatment-naive HIV-1-infected individuals.

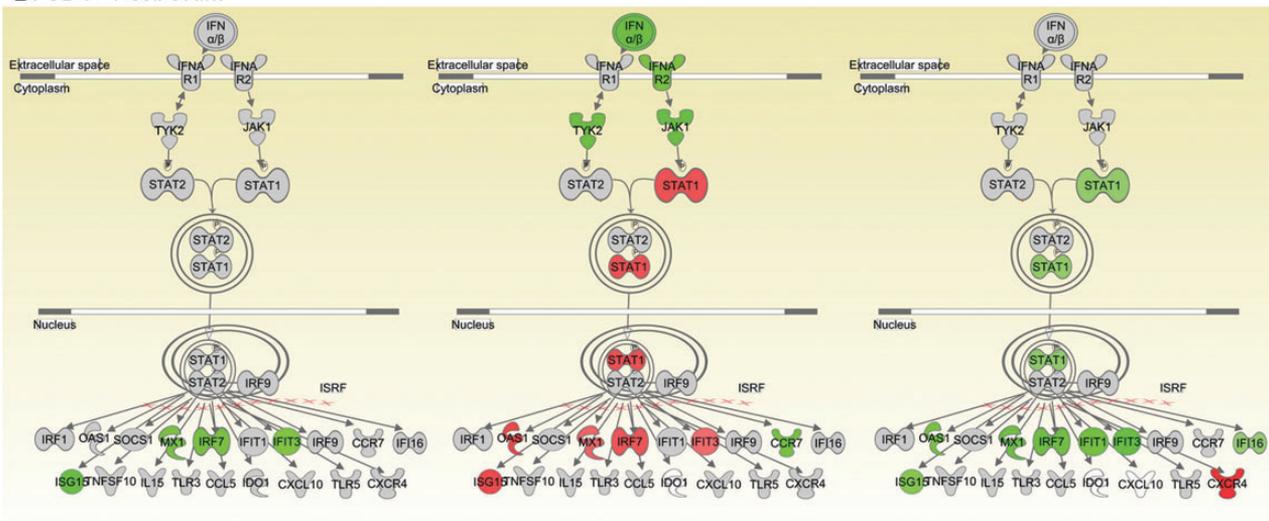
### Higher Expression of ISGs and Other Genes in Females After Adjusting for HIV-1 Viral Load

Sex differences in HIV-1-associated gene expression levels were subsequently analyzed by comparing gene expression profiles for cells derived from females (*n* = 19) with those from

### A. HIV-1 viral load



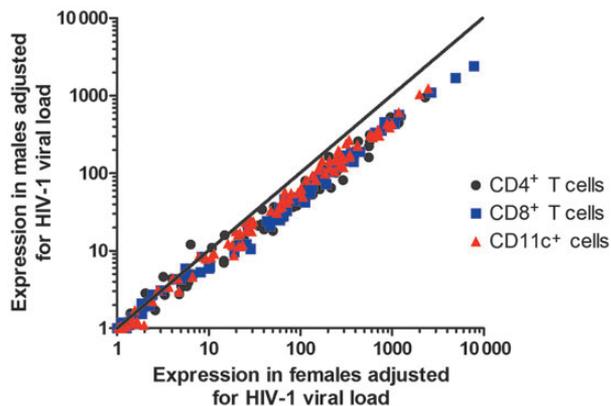
### B. CD4+ T cell count



**Figure 1.** Associations between gene expression and human immunodeficiency virus (HIV)-1 clinical parameters. Peripheral blood mononuclear cells from treatment-naive chronically HIV-1-infected individuals were sorted into CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD11c<sup>+</sup> cells. All 3 cell types were lysed, and mRNA expression was analyzed using Nanostring technology. Genes downstream of interferon-alpha stimulation, including the Jak/Stat pathway and ISGs, were examined in association with (A) HIV-1 viral load and (B) CD4<sup>+</sup> T-cell count. The mRNA expression levels are shown for CD11c<sup>+</sup> cells (left), CD4<sup>+</sup> T cells (middle), and CD8<sup>+</sup> T cells (right), with those found to be significantly positively (red) or negatively (green) correlated to HIV-1 viral load or CD4<sup>+</sup> T-cell count highlighted. Genes in gray were not significantly correlated to the clinical parameters examined.

males ( $n = 19$ ). Direct comparison of the mean expression of each gene after normalization to housekeeping genes showed no significant differences between the sexes. However, when the results were normalized by the HIV-1 viral load, a trend toward higher gene expression levels was observed in all 3 cell types isolated from females, with differences being significant after adjusting for FDR in CD4<sup>+</sup> T cells ( $P < .001$ ) and CD8<sup>+</sup> T cells ( $P = .008$ ; Figure 2). Examination of specific genes demonstrated that several had higher expression levels in cells derived from females (Table 2); in particular, the IFN-stimulated gene ISG15 trended toward a higher expression level in females

than in males in all 3 cell types examined (Figure 3). After adjusting for FDR, however, the sex-based differences were no longer significant. In order to independently reconfirm the Nanostring results, we used qRT-PCR analysis to quantify the specific gene expression levels in the sorted patient samples. The expression levels of ISG15 measured by qRT-PCR were significantly higher in females as compared to males after adjusting for HIV-1 viral load (Figure 3, bottom graphs). As shown in Figure 3, the same levels of ISG15 mRNA expression in CD4<sup>+</sup> and CD8<sup>+</sup> T cells induced in males by  $10^5$  copies HIV-1 RNA per mL plasma were observed at 1.87 and 2.38 log lower



**Figure 2.** Higher gene expression levels in females than in males. The mean expression levels of each gene in females (n=19) and males (n=19) after normalization to housekeeping genes and normalized by human immunodeficiency virus-1 viral load are shown for CD4<sup>+</sup> T cells (black circles), CD8<sup>+</sup> T cells (blue squares), and CD11c<sup>+</sup> cells (red triangles). The line represents where the gene expression levels are equal in both genders. Higher gene expression in females compared to males was observed in all 3 cell types examined, with differences been significant after adjusting for false discovery rate in CD4<sup>+</sup> T cells ( $P < .001$ ) and CD8<sup>+</sup> T cells ( $P = .008$ ).

HIV-1 viremia in females ( $1.3 \times 10^3$  and  $4.2 \times 10^2$  copies/mL of HIV-1, respectively).

In addition to ISG15, expression levels of other genes were also found to be differentially affected by sex after adjusting for HIV-1 viral load (Table 2). In CD8<sup>+</sup> T-cell populations derived from females, the expression levels of the ISGs, MX1 and TNFSF10, also trended toward a higher level compared to males in the Nanostring analysis. Apart from the upregulation of ISGs in cells derived from HIV-1-infected females, we also observed an unexpected tendency for increased expression of CCR5 (in CD11c<sup>+</sup> cells), TLR2 (in CD11c<sup>+</sup> cells), and TLR5 (in CD4<sup>+</sup> T cells) derived from females as compared to males after adjusting for HIV-1 viral load ( $P = .015$ ,  $P = .047$ , and

$P = .039$ , respectively). Similar to ISG15, we also quantified the expression levels of CCR5 mRNA in CD11c<sup>+</sup> cells and MX1 mRNA in CD8<sup>+</sup> T cells using qRT-PCR, which confirmed the Nanostring results with significant sex-dependent differences (Supplementary Figure 1B). Taken together, these data show concordant results using Nanostring technology and qRT-PCR in demonstrating higher expression levels of some ISGs (ISG15 and MX1) as well as CCR5 in females compared to males after controlling for HIV-1 plasma viremia.

### Immune Activation Levels Correlated to Expression Levels of ISGs

The percentage of T cells expressing the activation markers CD38 and HLA-DR is a strong predictor of the speed of HIV-1 disease progression [17] and has been shown to be significantly higher in females than in males in a cohort of treatment-naive HIV-1-infected individuals after adjusting for HIV-1 viral loads [6]. We therefore assessed whether ex vivo expression levels of ISGs were associated with the level of T-cell activation in our study subjects. The expression levels of several ISGs, including IRF7, MX2, OAS1, and ISG15, which were associated with CD4<sup>+</sup> T-cell count or HIV-1 viral load, were also found to be associated with the percentage of CD38<sup>+</sup> HLA-DR<sup>+</sup> CD8<sup>+</sup> T cells, as measured by ex vivo flow cytometry staining (Table 3). Taken together, these data establish a significant association between differences in HIV-1-driven IFN- $\alpha$  production, upregulation of several ISGs, and T-cell immune activation in treatment-naive individuals during chronic HIV-1 infection.

## DISCUSSION

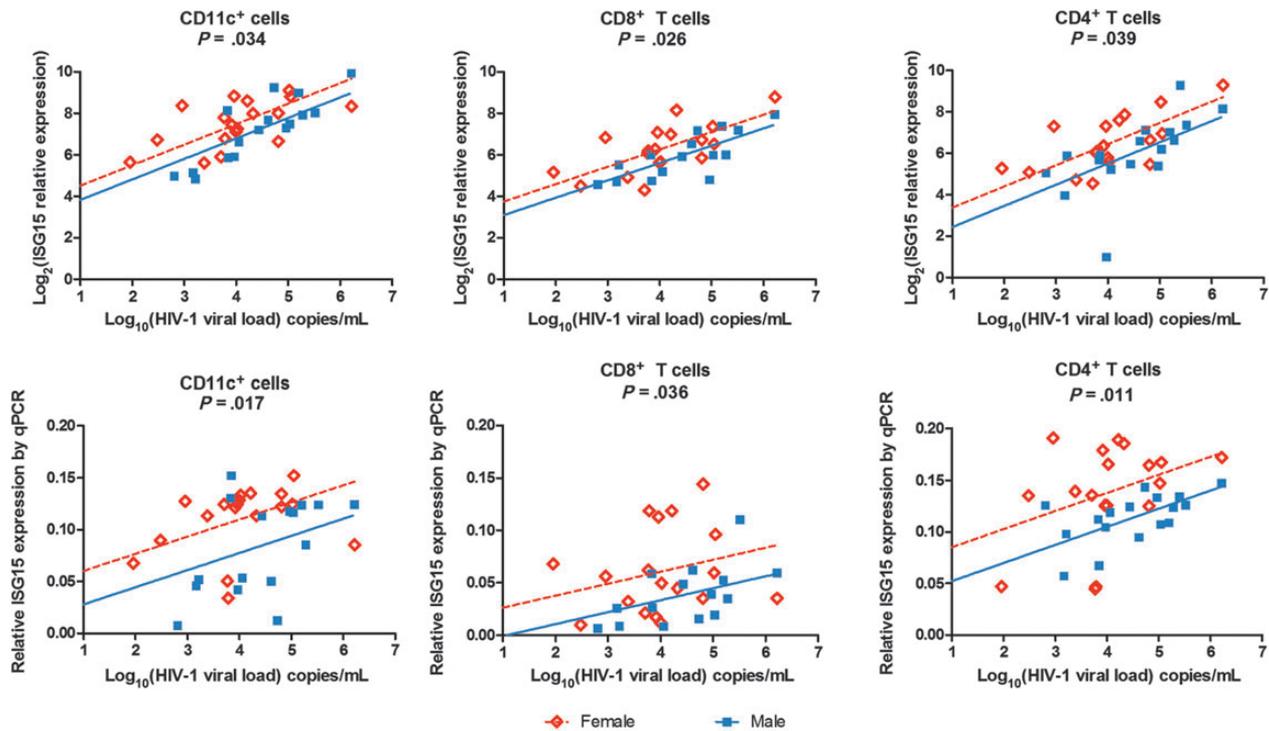
Previous work has demonstrated that there are differences between females and males in TLR signaling pathways and resulting type I IFN production [6, 12]. It has been suggested that these differences might account for sex variances in autoimmune diseases [18], responses to vaccinations [19], and viral

**Table 2. Sex Differences in Gene Expression**

Cell	Gene	Sex Difference (P value) <sup>a</sup>	Mean log <sub>2</sub> Male (gene expression level)	Mean log <sub>2</sub> Female (gene expression level)	Sex ANCOVA P value Adjusted for Baseline HIV-1 RNA <sup>a</sup>	Sex With Higher Expression
CD11c	CCR5	.094	3.253	3.863	0.015	Female
CD11c	ISG15	.537	7.213	7.498	0.034	Female
CD11c	TLR2	.093	7.046	7.700	0.047	Female
CD4	TLR5	.121	0.761	1.384	0.028	Female
CD4	ISG15	.388	6.003	6.468	0.039	Female
CD8	MX1	.211	7.306	7.718	0.018	Female
CD8	ISG15	.212	5.612	6.278	0.026	Female
CD8	TNFSF10	.131	4.762	5.308	0.044	Female

Abbreviations: ANCOVA, analysis of covariance; HIV, human immunodeficiency virus.

<sup>a</sup> Values not significant after adjusting for false discovery rate.



**Figure 3.** Higher expression of the interferon-stimulated gene (ISG) 15 in females than in males. ISG15 expression was higher in females (red open diamonds) as compared to males (blue closed squares) after normalization to housekeeping genes and adjusting for human immunodeficiency virus-1 viral load. The top 3 graphs demonstrate the data as measured by Nanostring technology, and the bottom 3 graphs demonstrate the data as measured by qualitative real time polymerase chain reaction (qRT-PCR). The data shown are the expression of ISG15 after normalization to housekeeping genes, and the line of best fit is shown for females (red dashed) and males (blue solid). The *P* values for the Nanostring analysis do not remain significant after adjusting for false discovery rate.

infections such as HIV-1 [6]. In the current study, we demonstrate that genes involved in the TLR and the type I IFN pathways, including MX1, IFIT3, IRF7, OAS2, ISG15, and IFIT1, are closely linked to HIV-1 viral load and CD4<sup>+</sup> T-cell count. Expression levels of a subset of these genes, including ISG15, MX1, and CCR5, were also found to be higher in treatment-naive HIV-1-infected females than in males after adjusting for HIV-1 viral load and to be associated with higher levels of immune activation in chronic HIV-1 infection.

Examination of gene expression in sorted cell subsets from treatment-naive chronically HIV-1-infected individuals allowed for the identification of several genes that were significantly associated with HIV-1 viral load and CD4<sup>+</sup> T-cell count. Consistent with previously published work [20], we observed that gene expression profiles were similar between CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HIV-1 infection, with the expression of 15 genes in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells being correlated to CD4<sup>+</sup> T-cell count. However, the pattern of gene expression in CD11c<sup>+</sup> cells significantly differed from that in T cells, with only 7 genes being associated with CD4<sup>+</sup> T-cell count in both CD11c<sup>+</sup> cells and T cells (*P* = .037; Supplementary Tables 1 and 2).

This further adds to previous data that demonstrate that the use of total PBMCs can mask variations in gene expression profiles of specific cell populations [15]. Despite differences between cells subsets, several ISGs (ISG15, MX1, IFIT3, IRF7, and OAS3) were positively correlated to HIV-1 viral load in all 3 cell types examined, supporting the hypothesis that HIV-1 viral load drives IFN- $\alpha$  production and hence higher ISGs expression levels. While some of these ISGs have yet undefined roles in HIV-1 disease and control, ISG15 has been described to interrupt the ubiquitination of Gag and its association with the protein Tsg101, thereby disrupting the release of HIV-1 virions [21, 22]. Another ISG, IRF7, is a master regulator of type I IFN transcription and has also been shown to be upregulated in HIV-1 [10, 23, 24] and to possibly play a role in the balance between tolerance and pathogenesis in primate simian immunodeficiency virus infections [25]. In addition to these 5 ISGs, other ISGs were also associated with HIV-1 viral load, but only in specific cell subsets. One potential reason for the variation might be differences in the kinetics of gene expression of individual ISGs, with some requiring less IFN- $\alpha$  to be activated. Furthermore, not all IFN- $\alpha$  subtypes are equal in their

**Table 3. Genes Associated With CD8<sup>+</sup> T-cell Expression of CD38 and HLA-DR**

Cell	Gene	<i>R</i>	<i>P</i> Value <sup>a</sup>
CD11c	CD38	0.440	.009
CD11c	IFNA5	0.379	.027
CD11c	TLR3	0.363	.035
CD11c	EIF2AK2	0.347	.044
CD4	STAT1	0.513	.002
CD4	OAS3	0.459	.006
CD4	TRIM5	0.453	.006
CD4	MX2	0.452	.006
CD4	IRF7	0.451	.007
CD4	TLR5	0.439	.008
CD4	MX1	0.430	.010
CD4	CD38	0.405	.016
CD4	OAS1	0.404	.016
CD4	TNFSF10	0.394	.019
CD4	MKI67	0.387	.022
CD4	FOXP3	0.370	.029
CD4	MYD88	0.354	.037
CD4	ISG15	0.349	.040
CD4	EIF2AK2	0.346	.041
CD4	TLR1	0.345	.042
CD4	IL1R1	0.337	.047
CD4	IFIT1	0.334	.0495
CD8	CD38	0.507	.002
CD8	CLEC7A	-0.468	.005
CD8	STAT1	0.412	.016
CD8	CCR5	0.411	.016
CD8	TYK2	-0.388	.023
CD8	OAS2	0.372	.030
CD8	TLR4	0.355	.040
CD8	MYD88	0.348	.043
CD8	CXCR4	-0.347	.044

<sup>a</sup> Values not significant after adjusting for false discovery rate.

function [26, 27]. Therefore, differences in the IFN- $\alpha$  subtypes produced may also account for why only some specific ISGs were found to be associated with HIV-1 viral load.

Direct comparison of gene expression levels between cell subsets showed no significant differences between the sexes and is in line with previous data demonstrating that differences in HIV-1-associated immune activation and disease progression between females and males were not observed when viral load level was not taken into account [4, 6, 7]. However, after adjusting for HIV-1 viral loads, the expression levels of several genes, including a subset of ISGs (MX1, ISG15 and TNFSF10), were found to differ between females and males as measured using the Nanostring technology and reconfirmed to be statistically significant using qRT-PCR. The qRT-PCR results demonstrated that 1.87 and 2.38 log<sub>10</sub> less plasma virus per mL was

required in females to stimulate the same levels of ISG15 mRNA expression in CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, respectively, as observed in males. These results are consistent with previously published work using a larger ACTG cohort, demonstrating that females with approximately 1 log<sub>10</sub> less of plasma HIV-1 RNA have the same level of CD8<sup>+</sup> T-cell immune activation as observed in males [6]. Taken together, these data show that the expression of some ISGs, including MX1 and ISG15, is significantly higher in females as compared to males after controlling for the level of viral replication and may contribute to the sex-dependent differences in immune activation levels observed in chronic HIV-1 infection.

Other genes were also found to be expressed differentially based on sex, including TLR2, TLR5, and CCR5. TLR2 recognizes bacterial lipopeptide and TLR5 recognizes flagellin and have not been shown to directly recognize HIV-1-encoded ligands. The upregulation of TLR2 and TLR5 could potentially be the result of secondary opportunistic infections or systemic immune activation in response to microbial translocation [28]. Although it has not been shown that IFN- $\alpha$  directly upregulates TLR2, there is evidence showing increased TLR2 gene expression in individuals with systemic lupus erythematosus, an autoimmune disease that is linked to TLR7/IFN- $\alpha$  activation, suggesting that IFN- $\alpha$ -associated activation alters TLR2 expression [29]. An increase in TLR2 might have detrimental effects in HIV-1 infection as engagement of this receptor has been shown to increase HIV-1 transmission from DCs to CD4<sup>+</sup> T cells [30]. Another factor that might play a role in the described faster disease progression in females is the observation of higher expression of CCR5, a coreceptor required for HIV-1 entry in CD11c<sup>+</sup> cells derived from females after adjusting for HIV-1 viremia. These data show that several genes that have described roles in HIV-1 pathogenesis also have a trend toward higher expression levels in HIV-1-infected females compared to males and may contribute to sex-based differences in HIV-1 disease progression.

One limitation of this study was that gene expression was quantified only using cells collected from the peripheral blood, and differences that might occur in various tissue compartments such as the gut-associated lymphoid tissue, which serves as a major site for HIV-1 replication, would have been missed. Although previous work has suggested that pDCs in the tissue may not retain the function of IFN- $\alpha$  production [31], other studies have shown that expression of some ISGs and TLRs are still elevated in the lymphatic tissue during HIV-1 infection [32]. Another caveat of this study was the use of sorted cell subsets that might have been activated during the cell-sorting process. Precautions were taken to minimize the effects of cell sorting on mRNA transcription levels by keeping all samples at 4°C or on ice. Despite these potential limitations, significant gender differences in the expression of several ISGs were observed using blood samples, suggesting an overall strong effect of sex on the response of the type I IFN pathway to HIV-1.

Taken together, this study provides novel evidence that sex-specific differences in IFN- $\alpha$  responses to HIV-1 are associated with sex-specific differences in CD8<sup>+</sup> T-cell immune activation in chronically HIV-1-infected individuals, potentially resulting in the described faster HIV-1 disease progression in females compared to males with the same viral load [4, 7]. These findings accentuate the importance of sex-dependent differences in the expression of a number of genes involved in the immune response and are in line with sex differences recently characterized in other disease models as well as in vaccine studies [19, 33, 34]. Further study of the mechanisms underlying these sex-based differences, both those that are associated with IFN- $\alpha$  and those that are not, will shed light on the factors that affect the outcome of several diseases, including infectious diseases and autoimmunity, and might allow for the development of novel interventions to target the pathways involved.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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