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CHARACTERIZATION OF THE FUNCTIONS INDUCED BY INTERFERON-λ3 IN HUMAN PLASMACYTOID DENDRITIC CELLS

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ABBREVIATIONS

ADA	adalimumab
BDCA	blood dendritic cell antigen
BTK	Bruton’s tyrosine kinase
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
CD62L	L-selectin
CLRs	C-type lectine receptors
DCs	dendritic cells
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETA	etanercept
FACS	fluorescence-activated cell sorting
FITC	fluorescein
GAS	gamma-activated sequence
HCV	hepatitis C virus
HEVs	high endothelial venules
HIV	human immunodeficiency virus
ICOS-L	inducible co-stimulator ligand
IFIT1	Interferon-Induced Protein with Tetratricopeptide Repeats 1
IFNs	interferons
IRAK4	IL-1 receptor-associated kinase 4
IRF7	interferon regulatory factor 7
ISG15	ISG15 Ubiquitin-Like Modifier
ISGF3	IFN-stimulated gene factor 3
ISG	interferon stimulated gene
ISRE	interferon stimulated response element
ITIMs	intracellular tyrosine-based inhibitory motifs
JAK1	Janus kinase 1
LN	lymphnode
M‐CSF	macrophage colony stimulating factor
MAPK	mitogen-activated protein kinases

mDCs	myeloid dendritic cells
MHC	major histocompatibility complex
Mx1	Myxovirus Resistance 1
MyD88	myeloid differentiation primary response protein 88
NF-κB	nuclear factor-κB
NK	natural killer
PBMC	peripheral blood mononuclear cells
PD-L1	programmed death ligand 1
pDCs	plasmacytoid dendritic cells
PGE_2	prostaglandin 2
PHH	primary human hepatocytes
PNAd	peripheral lymph node adressins
poly(I:C)	polyinosinic:polycytidylic acid
PRR	pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand-1
SLE	systemic lupus erythematosus
SNPs	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TAK1	TGF-β-activated kinase 1
TGF	transforming growth factor
Th	T helper
TLRs	toll like receptors
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TYK2	tyrosine kinase 2
USP18	ubiquitin carboxy-terminal hydrolase 18

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PREMISES

Type III interferon (IFN) family is composed by IFNλ1, IFNλ2, IFNλ3 and the recently discovered IFNλ4. IFNλs are antiviral cytokine whose main function is to counteract viral spreading and promptly initiate the antiviral response in an infected host. For several years, this important role was thought to be peculiar of the well-known type I IFN family, composed by IFNα and IFNβ. Instead, both type I and type III IFN families elicit similar responses in cells expressing their specific receptors, activate similar signaling pathways and induce hundreds of interferon-dependent antiviral mediators. In the last years, after the discovery of IFNλs, increasing numbers of studies have detected their presence in the context of several viral-mediated pathologies affecting mainly the anatomic barriers and mucosal tissues. As an example, IFNλ3 contributions in modulating the immune response during HCV infection in the liver has been reported and highlighted by several important studies, even though its ultimate role during HCV pathogenesis remains not completely understood.

Plasmacytoid dendritic cells (pDCs) are one of the DC subsets that, among other functions, are highly specialized in the production of type I IFNs, thus promoting antiviral immune responses. In fact, pDCs rapidly and strongly respond to viral particles and nucleic acids *via* potent secretion of IFNα, and subsequently present the captured viral antigens to T cells initiating adaptive immune response. More recently, pDCs have been shown to produce also IFNλs upon treatment with different types of viruses, coculture with HCV-infected cells or synthetic ligands for TLR7 and TLR9. Moreover, among leukocytes, only pDCs, and less prominently B cells, have been shown to express IFNλR, but only pDCs have been unequivocally shown to respond to IFNλs in terms of altered CD80 and MHC-I expression, STAT1 phosphorylation activation and MX1 mRNA induction.

In this study, I have extensively analyzed how human pDCs respond upon incubation with IFNλ3. My data not only confirm the pDC responsiveness to IFNλs, but also greatly extend previous observations already reported for IFNλ1. The purpose of my study has been to achieve a comprehensive and more complete characterization of pDC behavior in the presence of IFNλ3. This could give us some

important information on pDC peculiar skills, paving the way for further analysis on IFNλ-pDC crosstalk under specific context.

I. INTRODUCTION

1.1 PLASMACYTOID DENDRITIC CELLS (pDCs)

1.1.1 Properties of human pDCs

Within the heterogeneous dendritic cell (DC) family, two main subsets of blood DCs can be discriminated based on their phenotype and functional characteristics: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). The mDC subset can be further divided in BDCA1$^+$/CD1c$^+$ cells and BDCA3$^+$/CD141$^+$ cells. CD1c$^+$ DCs have been shown to readily stimulate naïve CD4$^+$ T cells and to secrete high amounts of IL-12 in response to toll-like receptor (TLR) ligation, whereas CD141$^+$ DCs do not secrete much IL-12 but are well equipped to take up dead and necrotic cells for subsequent cross presentation of derived antigens to CD8$^+$ T cells (1). In contrast to mDCs, pDCs have a very different protein expression profile reflecting their important and unique function in the secretion of type I Interferon (IFNα and IFNβ) and in the anti-viral immune response (1).

pDCs are rare cell type constituting only 0.2% to 0.6% of of peripheral blood cells in healthy individuals. pDCs were originally described in human lymph nodes (LNs), and are mostly known for their ability to quickly produce large amounts of IFNα following viral infection, implicating pDCs as an important contributor during the early phase of anti-viral response through induction of hundreds of interferon stimulated genes (ISGs)(2,3).

→ pDC phenotype

Human pDCs were first isolated from human blood and tonsils as CD4$^+$ CD123$^+$ HLA-DR$^+$ cells (4,5). In general, human pDCs phenotypically lack lineage markers for B and T cells, such as CD19 and CD3, myeloid and classical DC markers CD14, CD16 and CD11c, and natural killer (NK) cell marker CD56 (6). Several relatively pDC-specific surface markers have been established, such as BDCA2/CD303 and ILT7 (immunoglobulin like transcript 7); other useful markers include human CD123 and BDCA4/neuropilin-1 (6,7). BDCA2 is a member of the C-type lectin family of transmembrane glycoproteins that is specific for pDCs; ILT-7 belongs to the leukocyte immunoglobulin-like receptor gene family; CD123 is

the IL-3 receptor-α (IL-3Rα) chain and BDCA4 is a receptor for members of the semaphorin family (8,9). Peculiar surface markers of the major human DCs populations are depicted in **Figure 1**.

Adapted from Collin M. et al., Immunol, 2013; 140: 22–30

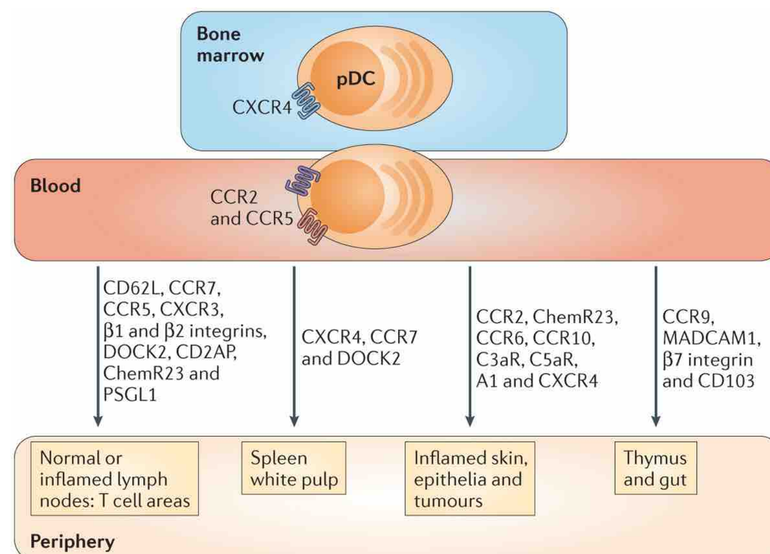
Figure 1. Surface markers of the major human DC populations

→ *Trafficking of pDCs*

pDC migration from the blood stream to the periphery is quite different from that of mDCs. mDCs typically seed peripheral tissues and become resident cells that migrate into T cell-rich areas of lymphoid organs through afferent lymphatics. On the contrary, pDCs released from the bone marrow into the blood stream reach T cell areas of the LNs mainly through high endothelial venules (HEVs)(3,4,10). This migration appears to be associated with their selective expression of CD62L (L-selectin) and chemotactic receptors C-C chemokine receptor type 7 (CCR7), which interact sequentially with peripheral lymph node adressins (PNAd) and chemokines CCL19 and CCL21 constitutively expressed by HEVs and stromal cells (9,11). In the LNs, pDCs have been found in close contact with T lymphocytes, Natural Killer T (NKT) cells, B lymphocytes, and NK cells (11,12).

pDCs are difficult to detect in most peripheral tissues in resting conditions. However, large numbers of pDCs have been found in several tissues during viral infections, such as in skin infected with varicella zoster virus, human papilloma virus, or in the small intestine under both normal and inflammatory conditions. At

sites of infection pDCs can activate or get activated by interaction with other immune cells or by soluble factors (8,13,14). In contrast to mDC studies, reports addressing which inflammatory chemokines and adhesion receptors specifically drive migration of human pDCs are scarce. Human pDCs express chemotactic receptors chemokine (C-X-C motif) receptor 3 (CXCR3), a receptor for inflammatory chemokines CXCL10 (IP-10), CXCL11 (ITAC), and CXCL9 (MIG), as well as CXCR4, a receptor for CXCL12 (SDF-1), that likely mediate recruitment of pDCs into lymphoid organs and/or into inflamed tissues (10,15,16). pDC migration involves also P-selectin glycoprotein ligand 1 (PSGL1), β1 and β2 integrins, and multiple chemokine receptors such as CCR2, CCR5, CCR6, CCR7, CCR9 and CCR10, as shown in **Figure 2** (17,18).



From Swiecki M. et al., Nat. Rev. Immunol. 2015; 15: 471–485

Figure 2. Factors influencing pDC migration

→ *pDCs as a source of type I IFNs in viral infections*

Although constituting only 0.2–0.6% of human blood cells, pDCs produce over 80% of IFNα among peripheral blood mononuclear cells (PBMC) in response to many viruses (2). Within 6 hours of activation, human pDCs dedicate 50% of the induced transcriptome to type I IFN genes (6,19). Type I IFN induces a global

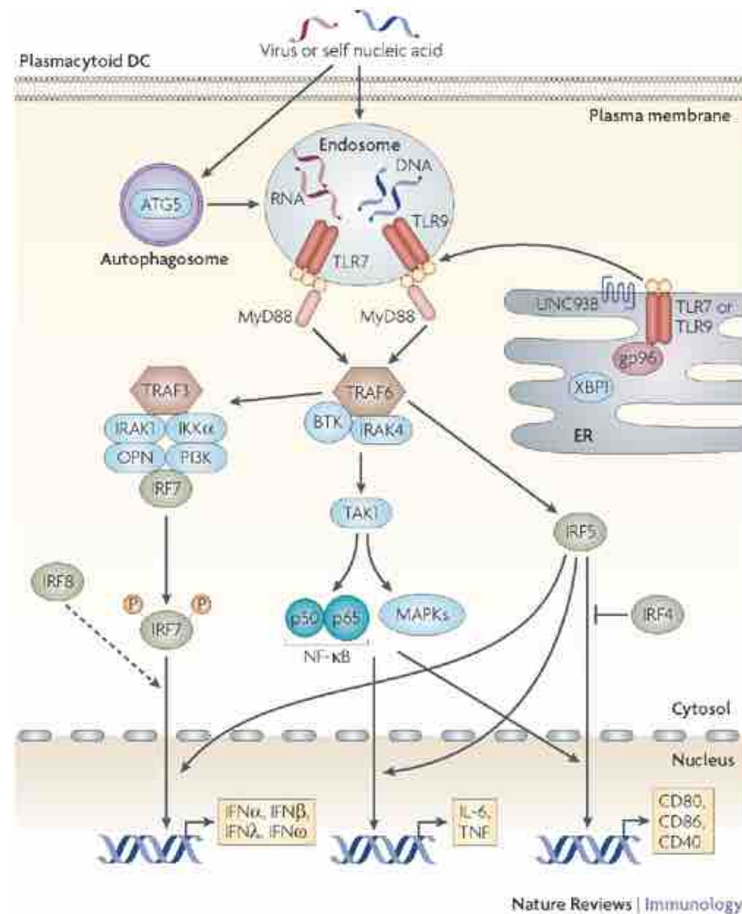
response that limits the spread of viral infections through production of antiviral factors, but also initiates a network of cellular and molecular events that are crucial to the generation of protective immune responses (20). More recently, it has also been shown that pDCs produce type III IFNs (namely IFNλ1, IFNλ2 and IFNλ3) upon treatment with different type of viruses (more details discussed below)(21).

pDCs produce other proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor α (TNFα), which regulate T, B, and NK cell and mDC responses and activity (22,23). In addition, pDCs produce several distinct chemokines such as CCL4, CCL5, CXCL9 and CXCL10, that allow a coordinated attraction of different immune effectors to the site of infection (17,24,25).

→ pDCs sense viruses through TLR7 and TLR9

The ability of pDCs to quickly secrete enormous amounts of IFNs, proinflammatory cytokines and chemokines depends on cellular sensors that promptly detect the presence of viral DNA and RNA. The recognition of viruses or self nucleic acids by pDCs is mainly mediated by TLR7 and TLR9, which are located in endosomal compartments (26). Activation of these receptors in pDCs results in their secretion of type I IFNs via the myeloid differentiation primary response protein 88 (MyD88)-Interferon regulatory factor 7 (IRF7) pathway, as well as their production of pro-inflammatory cytokines and chemokines via the MyD88-nuclear factor-κB (NF-κB) pathway (26,27). Intracellular nucleic-acid sensors and signaling pathway is depicted in **Figure 3**.

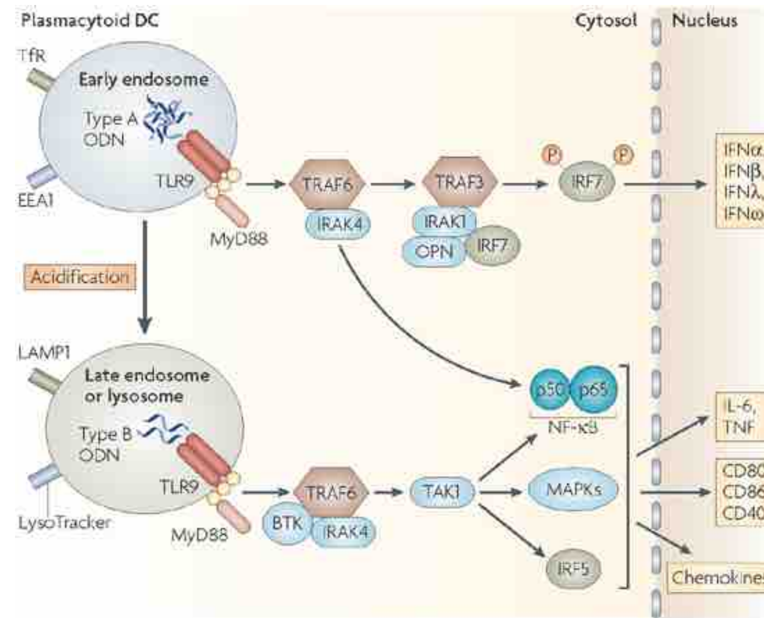
TLR9 accounts for pDC responses to unmethylated CpG motifs or synthetic oligonucleotides, such as CpG-ODN, which mimic bacterial or viral DNA(28) . TLR7 is responsible for pDC responses to guanosine or uridine-rich, single-stranded RNA from viruses or synthetic analogs such as Imiquimod (R837) (29). In addition to TLRs, pDCs express several C-Type Lectin Receptors (CLRs), including BDCA2, DEC-205, dectin-1 and DCIR (DC immunoreceptor), but also Fc receptor CD32 (FcγRII) and high-affinity IgE receptor (FcεRI) (16,30).



From Gilliet M. et al., Nat. Rev. Immunol. 2008; 8:594-606

Figure 3. Activation pathways in pDCs responding to nucleic acids

pDCs show differential responses based on the type of virus/bacteria or synthetic agonists that are recognized, which has been suggested to be attributed to a different site of TLRs activation within the endosomal system (31). As shown in **Figure 4**, multimeric CpG-A oligonucleotides (known also as type A ODN) aggregate in early endosomes where they seem to preferentially activate the MyD88-IRF7 pathway that induces type I IFNs (32). By contrast, monomeric CpG-B (known also as type B ODN) is transferred quickly to an endolysosomal compartment where it activates preferentially the MyD88-NF-κB pathway that triggers the expression of co-stimulatory molecules (i.e., CD40, CD80, CD86) and the secretion of pro-inflammatory cytokines and chemokines (33,34).



From Gilliet M. et al., Nat. Rev. Immunol. 2008; 8:594-606

Figure 4. Signaling of CpG ODN classes in different endosomal compartments

The activation of TLR7 and TLR9 by nucleic acids leads to the the assembly of a multiprotein signal transduction complex in the cytoplasm, containing IL-1 receptor-associated kinase 4 (IRAK4), TNF receptor-associated factor 6 (TRAF6) and Bruton’s tyrosine kinase (BTK) (**Figure 3** and **4**) (8). Phosphorylation of IRF7 and its translocation into the nucleus initiate the transcription of IFNs genes (35). The rapidity of IFN production exerted by pDCs is mainly mediated by their constitutive expression of IRF7 (32). This allows the rapid assembly of the multiprotein signal transduction complex described above. Other cell types, including mDCs, do not express IRF7 constitutively but require its upregulation in response to IFN-β feedback signaling following virus-induced activation of IRF3 (35). TLR7/9 signaling pathways can lead also to ubiquitinylation of the protein kinase transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) that consequently activate NF-κB and mitogen-activated protein kinases (MAPK) (**Figure 3** and **4**) (17,32). Known NF-κB members are RelA/p65, RelB, cRel, p52, and p50, which form homo- or heterodimers (36). p65/p50 dimers are directly

responsible for expression of costimulatory molecules, whereas IRF5, together with MAPK activation, seems to be crucial for the production of IL-6 and TNFα (23,37).

→ ***Regulation of type I IFN responses by pDC receptors***

Given the importance of type I IFNs in activating a wide range of immune cells, IFN production by pDCs needs to be under tight control, to prevent aberrant immune responses that could harm the host (23). A number of surface receptors that modulate the type I IFN production by pDCs have been identified. Many of these receptors contain intracellular tyrosine-based inhibitory motifs (ITIMs). BDCA2 and ILT-7 both associate with the γ-chain of the FcεRI, activate pDCs through an immunoreceptor-based tyrosine activation motif (ITAM)-mediated signaling pathway (26,38), and suppress the ability of pDCs to produce type I IFNs in response to TLR ligands (17,39). Other receptors shown to inhibit type I IFN production by human pDCs include NKp44, CD300A and CD300C, DCIR and FcγRII (40–42).

→ ***pDCs as antigen-presenting cells (APCs)***

In addition to cytokine secretion, activated pDCs undergo a characteristic DC maturation program (5). Upon activation by viral particle and/or TLR agonists, in fact, pDCs upregulate major histocompatibility complex (MHC) and co-stimulatory molecules, ultimately leading to the differentiation of pDCs into mature DCs with the ability to stimulate naive T cells (43).

- *Immature pDCs*

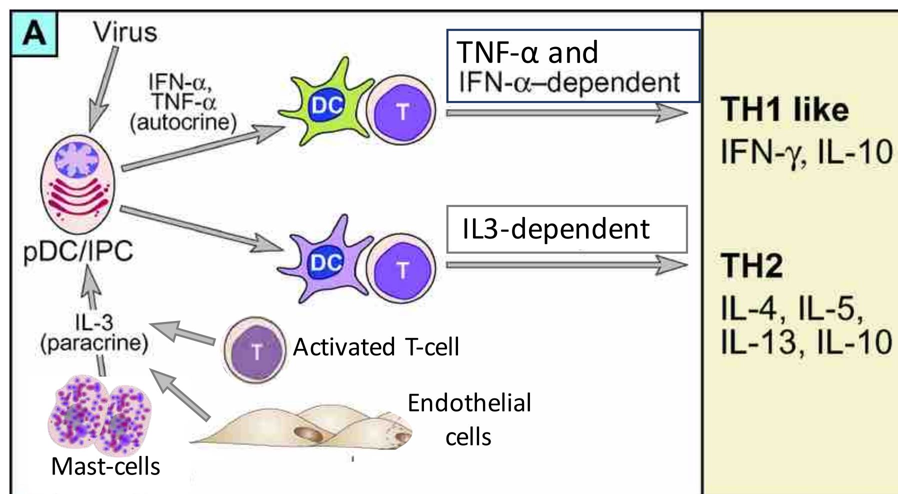
Nonactivated (immature) pDCs freshly isolated from the blood express low to undetectable levels of CD40, CD80 and CD86 and are therefore incapable of inducing significant proliferation of naive T cells (4,44). However, immature pDCs can induce antigen-specific anergy in $CD4^+$ T cell clones. In fact, despite the lack of costimulatory molecules, nonactivated pDCs constitutively express inducible co-stimulator ligand (ICOS-L) (45), which promotes survival and expansion of ICOS-expressing $FoxP3^+$ Tregs (46).These findings suggest a specialized role of nonactivated pDCs in peripheral tolerance (more details discussed below).

Accordingly, pDCs with an immature phenotype can suppress inflammatory responses to inhaled allergens and inhibit acute graft-versus host disease (8).

- *Mature pDCs*

Activated pDCs induce a broad spectrum of T cell differentiation (i.e., Th1, Th2, Th17, but also Treg) based on the cytokines secreted and cell surface proteins expressed, thus acting as immunogenic cells (**Figure 5**) (45,47,48).

Upon activation through TLR7 and TLR9, human pDCs differentiate into mature DCs. Auto/paracrine production of IFNα promotes pDC survival *via* induction of antiapoptotic genes, whereas TNFα supports pDC maturation (49). Several studies have demonstrated that activated pDCs mostly induce a Th1 phenotype (IFN-γ/IL-12) in response to CpG, TLR7 and/or viruses, but Th2 (IL-4) and Th17 (IL-17) skewing has also been reported when pDCs are activated with IL-3 plus CD40 or TLR7 ligands, respectively (50–52). IL-3 is a cytokine and growth factor that can be secreted *in vivo* by endothelial cells or activated T cells. pDCs mature into DCs in culture with IL-3 or IL-3 plus CD40L (4) and upregulate the costimulatory molecule OX40L, which leads to priming of T cells secreting Th2 cytokines IL-4, IL-5, and IL-10 (53). Finally, TLR activated pDCs express programmed death ligand 1 (PD-L1) (16,54), which may induce T cell anergy/suppress T cell activation by binding to its receptor, programmed cell death protein 1 (PD1) (55).These findings suggest that even mature pDCs could act to prevent excessive inflammation, thus avoiding damage to the host.



Adapted from Liu YJ, Annu. Rev. Immunol. 2005; 23:275-306

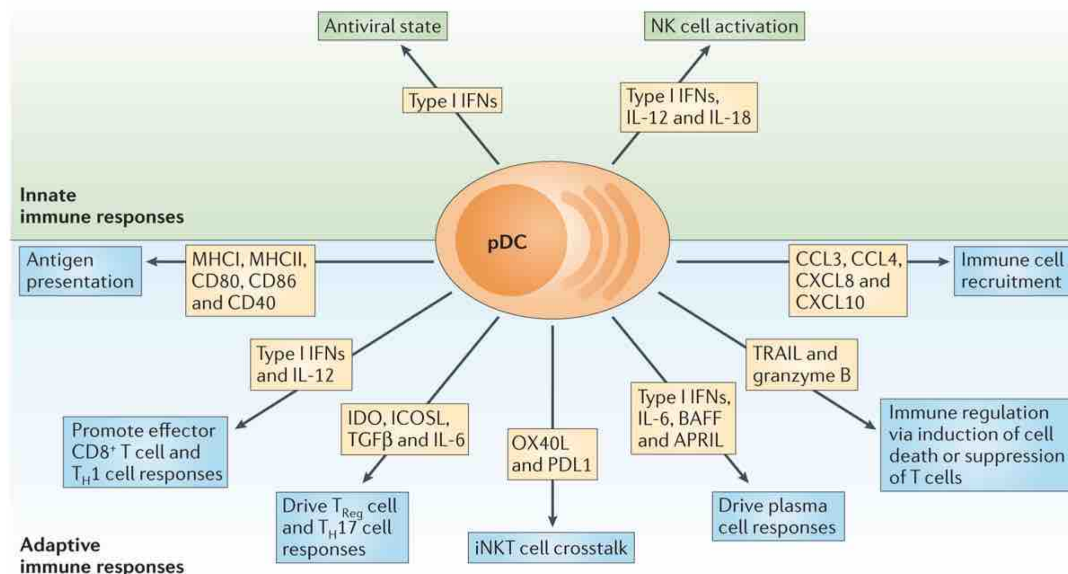
Figure 5. Functional plasticity of activated pDCs

→ *Antigen capture and presentation*

Numerous studies have established that pDCs are *bona fide* APC, capable of present antigens on both MHC class I and II molecules and thus trigger $CD8^+$ cytotoxic T cells and $CD4^+$ T cells. pDCs act also as professional APC in cross-presentation of exogenous Ag to $CD8^+$ T cells (56,57). Even if pDCs efficiently present endogenous antigens, they poorly present exogenous antigens when compared to mDCs (43). One of the reasons for this is that pDCs hardly take up exogenous antigens by phagocytosis or macropinocytosis (37,58). However, pDCs internalize certain exogenous antigens *via* specific receptor-mediated endocytosis, by BDCA-2, DEC-205, CD32 and DCIR (42,59).

1.1.2 Diverse functions of pDCs

As a major effector cell type in immunity, pDCs have been implicated in nearly all pathological immune responses. For example, important roles for pDCs have been suggested in allergy and asthma (60), antitumor immunity (61), and responses to both viral and nonviral pathogens (17,62). **Figure 6** shows pDC-mediated functions in both innate and adaptive immune responses.



From Swiecki M. et al., Nat. Rev. Immunol. 2015; 15: 471–485

Figure 6. Diverse functions of pDCs

→ *pDCs in viral infections: detection and reaction*

Type I IFN production by pDCs in response to acute viral infections is usually limited in time and amplitude (63). Secretion of type I IFNs is most evident at early time-points in systemic infections with viruses such as cytomegalovirus (CMV), vesicular stomatitis virus (VSV) and herpes simplex virus 1 (HSV1) and mediates an immediate containment of viral replication (17). Paradoxically, pDC responses to acute viral infections may not always be beneficial. Recent evidences indicate that excessive production of type I IFNs during influenza virus infection can result in uncontrolled inflammation and apoptosis of bronchial epithelium (64). Thus, the impact of pDCs on acute viral infections may vary considerably depending on the virus, the route of infection and the genetic background.

In humans, pDCs have been most extensively studied during human immunodeficiency virus (HIV) and chronic viral hepatitis, particularly hepatitis C virus (HCV) infections. The emerging picture suggests an important role for pDCs in these infections, although the exact mechanism and consequences of pDC activity are controversial at present (65). pDCs express CD4, CXCR4, and CCR5 and are therefore direct targets of infection by HIV, which uses these molecules to

infect T cells (10). Indeed, HIV^+ pDCs have been found in the thymus and tonsils of HIV-infected individuals (66). Depletion of pDCs from human thymocyte cultures enhanced HIV replication, suggesting that pDCs control HIV replication (67). In chronic hepatitis C virus patients, studies have shown that the number of circulating pDCs and their ability to produce type I IFNs are reduced, correlating with the persistence of the virus. It has been shown recently that pDCs can respond to HCV particles and particularly to HCV-infected hepatocytes through TLR7 (68,69). HCV may specifically impair pDC activity (70,71), thereby compromising T cell responses against it; however, other studies demonstrated normal pDC functionality on a per cell basis in chronic HCV (22,72). The resolution of this controversy would establish pDCs either as a weak link of anti-HCV immune response or as a potentially powerful effector cells that can be harnessed for immunotherapy.

→ ***pDCs in autoimmunity***

Despite the low frequency of pDCs in blood and lymphoid tissues, their high potential to produce IFNα in response to self-nucleic acids raised questions about their putative role in autoimmunity (73). Unwanted IFNα production by pDCs is involved in autoimmune pathogenesis, including systemic lupus erythematosus (SLE)(74), Sjogren's syndrome, and psoriasis (75). Blood and tissue cells of these patients have an IFN signature indicating that IFN-inducible upregulation of IFN-stimulated genes can be used as a disease biomarker (75).

Free self-nucleic are able to enter TLR containing endosomes when complexed with host derived factors that are aberrantly expressed in certain autoimmune diseases (76). In SLE, self nucleic acids are complexed with autoantibodies directed against nucleic acids or nucleoproteins, causing inflammation in the tissues. Nucleic acid–containing immune complexes trigger IFNα release from pDCs upon FcγRII-mediated uptake into endosomes and local engagement of TLR7/9 (77). As a result, pDCs are continuously activated to produce type I IFNs, leading to an unabated activation and maturation of other cell types such as mDCs that stimulate autoreactive T cells (77). Moreover, pDC-derived type I IFNs, together with IL-6, promote the differentiation of autoreactive

B cells into autoantibody secreting plasma cells (78). In psoriasis, free self-DNA forms complex with the cationic antimicrobial peptide LL37 overexpressed in skin lesions by activated keratinocytes (76). DNA complexed with LL37 enters endosomal compartments of pDCs and triggers high levels of type I IFN production via TLR9 in early endosomes (73,76) leading to a sustained pDC activation.

→ pDCs in tolerance

Non-lymphoid tissue pDCs, such as those residing in the airways, gut, and liver, play a significant role in regulating mucosal immunity and are critical for the development of tolerance to inhaled or ingested antigens (79). When pDCs are either unstimulated or alternatively activated, thus expressing ICOSL, OX40L, PDL1 and/or granzyme B, they promote tolerance to tumor cells, alloantigens and harmless antigens (17). Recent studies have also proposed that pDCs that capture antigens in peripheral tissues use CCR9 to migrate to the thymus where they induce deletion of antigen-specific thymocytes, contributing to immune tolerance (79).

→ pDCs in tumors

pDCs have been found in many solid tumors, including head and neck cancer, breast cancer, ovarian cancer, lung cancer, and skin tumors (61,80). In these tumors, pDCs are present in a nonactivated state and have been associated with the development and maintenance of the immunosuppressive tumor microenvironment (61,81). Mechanisms responsible for keeping the pDCs in this state include the secretion of prostaglandin 2 (PGE2) and TGFβ, which inhibit pDC-derived IFNα and TNFα production in response to TLR7 and 9 ligands, as well as CCR7 expression, thereby impairing the migration of pDCs to the tumor-draining LN to prime T cells with tumor antigens (61,81).

Unstimulated or alternatively activated pDCs can induce Treg cells through expression of indoleamine-pyrrole 2,3-dioxygenase (IDO) (82) or ICOSL (48). The accumulation of IDO-expressing pDCs in tumor-draining lymph nodes has been associated with worse clinical outcomes in patients with malignant tumors, including those with breast carcinoma (83). Human pDCs may also contribute to cancer progression via the production and release of the pro-apoptotic molecule

granzyme B (9,81) which suppresses T cell proliferation. In contrast to NK cells, pDCs do not release the pore-forming protein perforin and therefore are unable to kill target tumor cells by releasing lytic granules (84).

Conversely, pDCs can promote immunogenic antitumor responses if appropriately stimulated. Injection of activated pDCs loaded with tumor-associated peptides into patients with metastatic melanoma leads to favourable $CD4^+$ and $CD8^+$ T cell responses, indicating that vaccination using activated pDCs might be an attractive therapeutic strategy (85). TLR-activated pDCs in the tumor can stimulate NK cell activity and elicit potent $CD8^+$ T-cell-mediated antitumor immunity *via* cell-cell contact or indirectly *via* IFNα secretion (86). TLR-activated pDCs also upregulate the expression of TNF-related apoptosis-inducing ligand (TRAIL) (87,88) and acquire the ability to kill tumor cells *in vitro* (89), suggesting an additional mechanism by which activated pDCs may eventually induce antitumor activity.

1.2 THE BIG FAMILY OF IFNs

Discovered over 50 years ago, the interferons are historically best known for their ability to elicit viral resistance to cells (90). There are three distinct IFN families. The type I IFN family is a multi-gene cytokine family that encodes 13 partially homologous IFNα subtypes in humans, a single IFNβ and several poorly defined single gene products (IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ) (91). The type II IFN family consists of a single gene product, IFNγ, that is predominantly produced by T cells and NK cells, and can act on a broad range of cell types that express the IFNγ receptor (IFNγR) (92). The type III IFN family comprises IFNλ1, IFNλ2 and IFNλ3 and the recently identified IFNλ4 (93), which have similar functions to cytokines of the type I IFN family but restricted activity, as the expression of their receptor is largely restricted to epithelial cell surfaces (94).

1.2.1 Type I IFN

IFNα and IFNβ are the best-defined and most broadly expressed Type I IFNs. These cytokines are known for their ability to induce an antiviral state (95). First, they induce cell-intrinsic antimicrobial states in infected and neighbouring cells that limit the spread of infectious agents, particularly viral pathogens. Second, they modulate innate immune responses in a manner that promotes antigen presentation and NK cell functions. Third, they activate the adaptive immune system, thus promoting the development of high-affinity antigen-specific T and B cell responses and immunological memory (91,96).

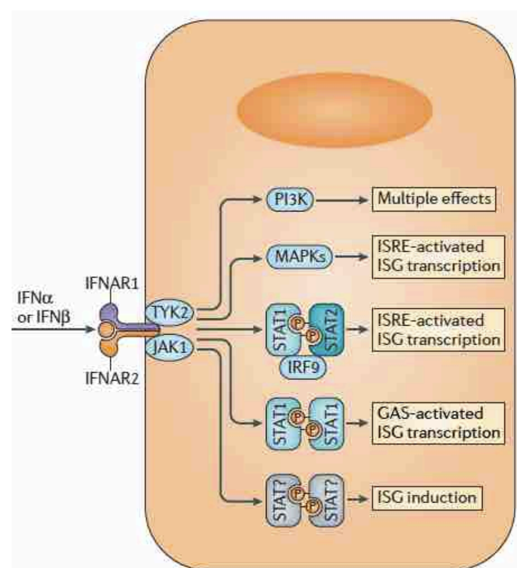
→ *Type I IFN production*

Almost all cells in the body can produce IFNα/β, and this usually occurs in response to the stimulation of pattern recognition receptors (PRRs) by microbial products (97). Diverse pathways downstream to these receptors transduce signals that converge on a few key molecules, such as the IRF family of transcription factors, that activate the transcription of genes encoding IFNα/β. The central tenet of IFNα/β production is that the IFNB gene is induced in an initial wave of transcription that relies on IRF3. 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 IFNα-encoding genes (98). NF-κB can be required as a cofactor (96,98). In pDCs, as previously mentioned, IFNα production is directly mediated by constitutive expression of IRF7 and to retention of the MYD88-IRF7 complex in endosomes.

→ *Type I IFN signaling and induction of ISGs*

IFNα and IFNβ bind a heterodimeric transmembrane receptor termed the IFNα receptor (IFNαR), which is composed of IFNαR1 and IFNαR2 subunits (99). In the canonical type I IFN-induced signaling pathway, IFNαR engagement was shown to activate the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate the latent cytoplasmic

transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2 (96,100), as shown in **Figure 7**. Tyrosine-phosphorylated STAT1 and STAT2 dimerize and translocate to the nucleus, where they assemble with IRF9 to form a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to its cognate DNA sequences, which are known as IFN-stimulated response elements (ISREs), thereby directly activating the transcription of hundreds of ISGs. The phosphoinositide 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway, NF-kB and MAPK pathways can also be activated downstream of IFNαR. This diversity of signaling pathways may in part explain the broad effects of IFNα/β, as it allows the transcription of a broad range of genes in addition to those dedicated to viral restriction (96,100). These include genes that encode cytokines and chemokines, antibacterial effectors, pro-apoptotic and anti-apoptotic molecules, and molecules involved in metabolic processes (101).



Adapted from McNab F. et al., Nat. Rev. Immunol. 2015; 15:87-103

Figure 7. Type I IFN receptor signaling

Several recent reports have extended our understanding of how the production of type I IFNs is regulated. Key new insights include:

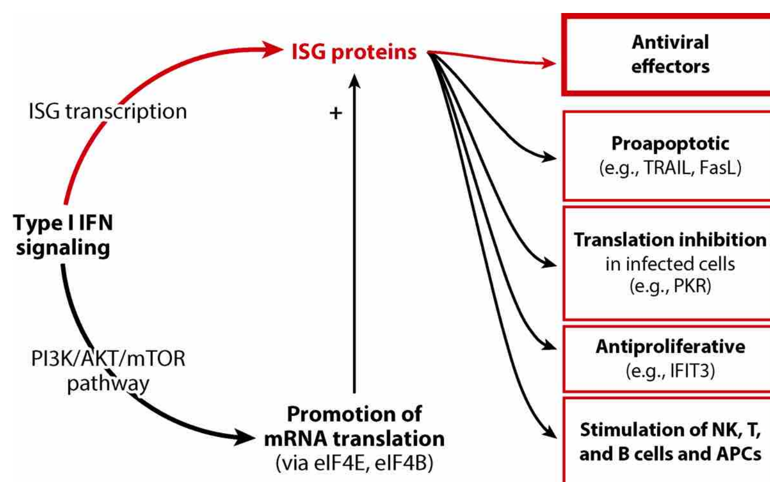
→ basal levels of type I IFN production under physiological conditions are

maintained by the commensal microbiota (102). Immune cells can respond rapidly to low levels of type I IFNs, a capacity that is maintained under homeostatic conditions by an autocrine loop in which small amounts of IFNα/β maintain basal expression levels of STAT1 and IRF9 (103). Basal IFNα/β expression and attendant tonic IFNαR signaling equips immune cells to rapidly mobilize effective antimicrobial programs.

→ Type I IFNs can be induced by host factors and cytokines such as TNFα, which signal via IRF1 rather than via IRF3 and IRF7 (104), and by macrophage colony stimulating factor (M‑CSF) (96).

→ The effects of type I IFNs: cell resistance and immune response

The ability of IFNs to restrict viral replication is largely attributable to the induction of ISGs by which IFNα/β promote an antiviral state (95,97). ISG-encoded proteins restrain pathogens by several mechanisms, including the inhibition of viral transcription, translation and replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism (105) (**Figure 8**). The fact that most viruses devote part of their limited genome to mechanisms that perturb IFNα/β production and/or IFNα/β-mediated signaling, thereby preventing ISGs from being induced, illustrates the importance of this cytokine family in host cell protection against viral infection (106).



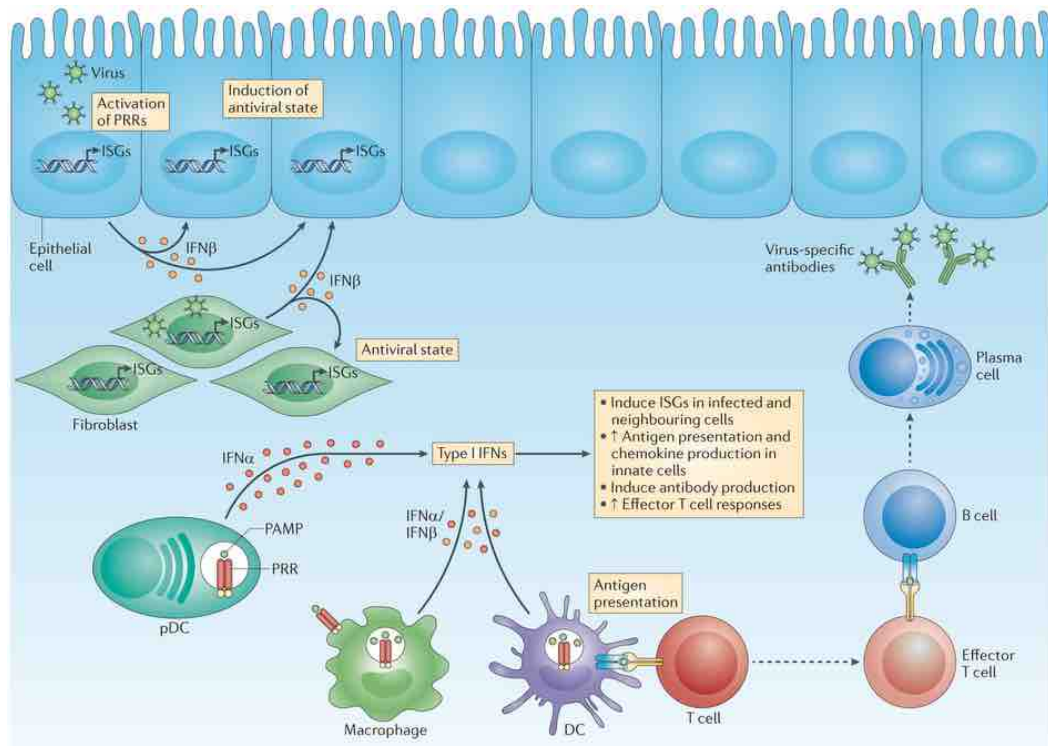
Adapted from Fensterl V. et al., Annu. Rev. Virol. 2015; 2:549–72

Figure 8. Interferon-stimulated genes: the mediators of the biological effects of IFNs

While it has been known for many years that type I IFNs promote resistance to viral infections, the impact of type I IFNs on immune cell functions is becoming increasingly appreciated. IFNα affect myeloid cells, B cells, T cells and NK cells, thereby enhancing the immune responses, more effectively resolving viral infections and improving the generation of memory responses (97). A summary of type I IFN-mediated function is depicted in **Figure 9.** In this context, IFNα have an activating effect on immature mDCs, enhancing the cell-surface expression of MHC class I and II molecules and co-stimulatory molecules, such as CD80 and CD86, which is associated with an increased ability to stimulate T cells (51,107). It has also been observed that IFNα promotes the ability of mDCs to cross-present antigens during viral infections (108). and the migration of mDCs to lymph nodes, through upregulation of chemokine receptors (109).

Several studies have revealed that DC turnover is strongly influenced by IFNα/β in vivo. IFNα regulates mDC and pDC numbers in vivo by inducing the downregulation of anti-apoptotic molecules, upregulation of pro-apoptotic molecules and caspase activation (110).

IFNα/β together with IL-12 augments NK cell and $CD8^+$ T cell cytolytic activities and IFNγ production *in vitro* and *in vivo*, promotes Th1 polarization of $CD4^+$ T cells, as well as long-term T cell survival and memory. Moreover, differentiation of B cells into immunoglobulin secreting plasma cells by IFNα/β is crucial for the development of local humoral responses against viruses (110).



From Ivashkiv L. et al, Nat. Rev.Immunol. 2014; 14:36-49

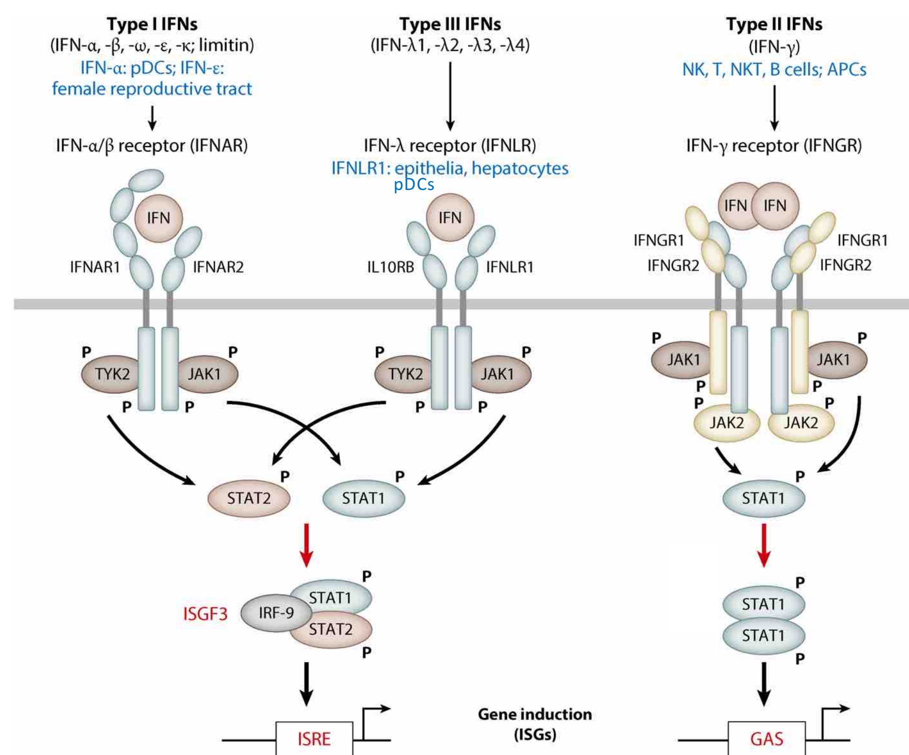
Figure 9. Type I IFN controls innate and adaptive immunity and intracellular antimicrobial programs

1.2.2 Type II IFN

IFNγ is the sole type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. IFNγ is produced primarily by $CD4^+$ and $CD8^+$ T lymphocytes and NK cells (111). There is now evidence that other cells, such as B cells and professional APCs, secrete IFNγ, to induce local cells activation (112). IFNγ production is controlled by cytokines secreted by APCs, most notably IL-12 and IL-18 (113). The main function of IFNγ is macrophage activation, rendering them able to exert its microbicidal functions. Macrophage recognition of many pathogens induces secretion of IL-12 and other chemokines [e.g., macrophage-inflammatory protein-1 (MIP-1)]. These chemokines attract NK cells to the site of inflammation, and IL-12 promotes IFNγ synthesis in these cells, thus inducing IFNγ-mediated macrophages activation. IFNγ induces the transcription of more than 200 genes,

including those for the production of antimicrobial molecules such as oxygen free radicals and nitric oxide, which represent one of the best effector mechanisms for elimination of bacteria (114). Other cellular effects of IFNγ include induction of an antiviral state, inhibition of cellular proliferation and effects on apoptosis, immunomodulation, and leukocyte trafficking (111).

Unlike the type I IFNs, which all appear to signal as monomeric cytokines, IFNγ signals as a homodimer receptor complex composed by two chains of each of the high-affinity (IFNγR1) and low-affinity receptors (IFNγR2) (99,115). In canonical IFNγ signaling, ligand engagement of the IFNγ receptor leads to activation of receptor-associated JAK1 and JAK2 and phosphorylation of STAT1. STAT1 homodimer translocates to the nucleus, binds to a regulatory DNA element termed gamma-activated sequence (GAS), and stimulates transcription of STAT1 target genes (**Figure 10**). Besides the JAK/STAT pathway, type II IFN can also activate other signaling pathways, including the MAPK, PI3K and the NF-κB pathway (116).



Adapted from Fensterl V. et al., Annu. Rev. Virol. 2015; 2:549–72

Figure 10. Ligand–receptor complex assembled by type I, II or III IFNs

1.2.3 Type III IFN

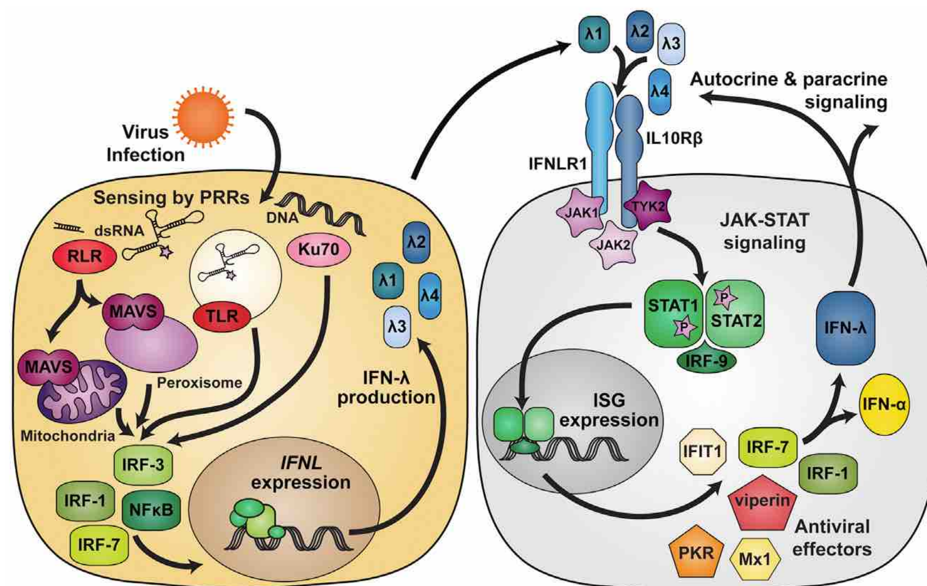
IFNλs were discovered by two independent groups (117,118). IFNλ family comprise four members: IFNλ1, IFNλ2, IFNλ3 and the very recently described IFNλ4 (93). Formally, IFNλs belong to the IL-10 family of cytokines containing IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26 (119,120). IFNλ2 and 3 share 96% sequence similarity, whereas IFNλ1 is less similar (121). IFNλ4 arises as a consequence of a frameshift mutation generating a new gene not normally expressed. It demonstrates only a 40.8% similarity to IFNλ3, acts through the same receptor and displays typical antiviral activity (93).

→ ***Expression of IFNλs***

Almost any cell type is able to express IFNλs in response to diverse viral infections. The stimuli that induce expression of IFNλ-encoding genes, including a range of viruses, are similar to those inducing expression of genes encoding IFNα/β (117,118,122,123). Nonetheless, there are differences in transcription factor requirements between IFNα/β and IFNλs. Initial characterization of promoter regions upstream of IFNλ1 and IFNλ3 identified binding elements for IRF-1, IRF-3, IRF-7, and NF-κB, and the combined activity of IRFs and NF-κB was required for maximal gene induction (124). Therefore, the pathways leading to type I or type III IFN gene expression is not entirely identical, type III IFNs being more dependent on NF-κB than type I IFNs (125).

Type III IFN can be expressed in a variety of primary human cell types of the hematopoietic lineage, such as monocytes and dendritic cells (126–128). Among non hematopoietic cells, epithelial cells are potent producers of type III IFNs (129). High levels of IFNλs were observed during viral infection of lung and liver tissues (122,130) and IFNλs seem to be the major IFNs induced in airway epithelial cells during infection with respiratory viruses (131). Within the HCV-infected liver both IFNα and -λs are present, but identification of the cell types that express IFNλs is difficult to determine. However, it is known that freshly isolated primary human hepatocytes (PHH) express IFNλs when infected with HCV (132) (**Figure 11**). Although there are no studies clearly addressing the issue of IFNλ production by DCs within the HCV-infected liver, it is known that DCs can secrete

IFNλs following *in vitro* stimulation (21,133–135). In this context, pDCs were shown to be important IFNλs producers upon viral infection with HSV-1, Sendai virus or cocolture with HCV-infected hepatocytes (21,127). However, in response to polyinosinic:polycytidylic acid [poly(I:C)] or after coculture with HCV infected cells, human CD141^{+} DCs were the major cell population producing IFNλs (134).



From Lazear H. et al., Immunity Rev. 2015; 43:15-28

Figure 11. Induction of IFNλ and IFNλ-activated signaling pathways

→ Restricted expression of IFNλR

The IFNλR consists of two subunits, IFNλR1 (alpha chain) and IL-10R2 (beta chain) (121). IL-10R2 shows a broad expression pattern (136) whereas, unlike the type I IFN receptor IFNαR, the IFNλR1 displays a restricted cellular distribution (**Figure 12**). Several studies examined the responsiveness of human cell lines and primary cells to IFNλs (99,135,137). Fibroblasts, splenocytes, bone-marrow derived macrophages and endothelial cells did not respond to IFNλs, although they responded to IFNα. Further studies demonstrated that epithelial cells are the primary targets of type III IFNs. Only organs with high-epithelial cell numbers express detectable levels of IFNλR (e.g., skin, intestine, hepatocytes and lungs)(129,138). Consistent with this pattern, the antiviral effects of IFNλs are most

evident against pathogens targeting epithelial tissues. Human hepatocyte cell line HepG2 and PHH express IFNλR and readily respond to IFNλs (139–141). Accordingly, IFNλ1 was shown to restrict H … and PHH (142).

Conflicting responses to IFNλs has b… …ells. Among hematopoietic cells, pDCs express the … and surface protein of IFNλR1 (21,134). It has l… …vely induces specific pDC response (134,143). I… …cells express mRNA of IFNλR1, such as B cells an… …data on protein expression and cell response to st… …ted. (141,144). On the contrary, no expression was … .

Responsive cells (human)
Cell lines
HepG2, HuH7 (hepatocytes)
HeLa
HaCaT (keratinocytes)
A594 (lung carcinoma cells)
Intestinal cell lines
Primary cells
Hepatocytes
Keratinocytes
Melanocytes
Natural killer cells
Airway epithelial cells
Nasal epithelial cells
Astrocytes
Neurons
pDCs
Monocytes
B lymphocytes
In vivo
Liver

NK cells

Nonresponsive cells (human and mouse)
Cell lines
Fibroblasts
Primary cells
Lymphocytes
Monocytes, macrophages
Endothelial cells
Fibroblasts
Adipocytes
Splenocytes
In vivo
Adipocytes
Endothelial cells

Hermant P. et al., J. Innate Immun. 2013; 30

Figure 12. Cells respon…

→ *IFNλ signaling an…*

Despite enga… receptors, the postreceptor signaling events after … exhibit remarkable overlap. The signaling pathways resemble that induced by type I IFNs (**Figure 10** and **11**) (119,135,145) and include JAK-family kinases activation, STAT1 and STAT2 phosphorylation, and association between activated STAT complexes and IRF-9 to form ISGF3, which translocates to the nucleus and induces expression of hundreds

of ISGs. Additionally, JAK2 phosphorylation is induced by IFNλs (146), suggesting that a distinct upstream signaling events might differentiate IFNλ from IFNα activity in a cell-dependent manner. In addition to activating STAT1 and STAT2, IFNλR ligand engagement can activate STAT-independent signaling cascades (MAPK and ERK) (147).

The transcriptional responses induced by IFNλ and IFNα are similar (148–150). No transcriptional signatures unique to IFNλs have been identified yet. However, the relative magnitude of gene expression induced by IFNα is often greater than that induced by IFNλs in many cell types. This may reflect a difference in the relative strength of signaling through type I IFN receptors versus type III IFN receptors. Alternatively, this difference may simply reflect a significant difference in the relative levels of expression of these receptors on the cell membrane (119). The IFNλ transcriptional response generally exhibits a delayed peak and longer duration (150). IFNα-treated Huh7 (Hepatocellular carcinoma cells) and PHH demonstrate a short induction of STAT1 phosphorylation (30 min-4 h), followed by a rapid peaked induction of ISGs mRNA. IFNλs, on the other hand, induces both a later and more sustained phosphorylation of STAT1 over 24 h and a slower increase in ISG expression (120,148,151). Of note, IFNλ3 demonstrates the highest anti-viral activity as measured by HepG2 challenge with encephalomyocarditis virus. Additionally, ISG induction [namely Myxovirus Resistance 1 (Mx1) and IRF9] by IFNλ3 was significantly higher compared to IFNλ1 and IFNλ2 (120,148).

The antiviral signaling is controlled by anti-inflammatory ISGs including USP18 (ubiquitin carboxy-terminal hydrolase 18) and SOCS1-3 (suppressor of cytokine signaling), which interfere with the STAT signaling cascade. They function as part of a negative feedback loop to limit the extent and duration of the IFN response (152). USP18 was shown to be necessary and sufficient to induce differential desensitization by impairing JAK1 at the IFNαR. The potent and sustained effects of USP18 upregulation in the context of a chronic infection such as HCV may significantly affect IFNα induced signaling, as USP18 desensitizes cells to further IFNα stimulation but does not inhibit IFNλ signaling (153). Moreover, SOCS1 negatively regulates type I IFN signaling via interaction with TYK2 but it has still to be demonstrated whether SOCS1 has a role also in the

regulation of type III receptor activation (153).

1.3 TYPE III IFNs AND pDCs: A CLOSE RELATIONSHIP

High levels of IFNλR1 have been detected on pDCs relatively to other cell populations in human PBMCs (21,144). In addition to high constitutive levels of expression, pDCs further up-regulated IFNλR1 mRNA after stimulation with TLR7 and TLR9 ligands, positioning the cell to respond rapidly to autocrine/paracrine IFNλ signals (143).

IFNλ1 treatment of PBMCs have limited effects in terms of induction of both ISG mRNA expression and proinflammatory mediator release, namely MCP-1, CXCL11 and IL-6 (154). This is mainly due to the restricted distribution of the IFNλR. In fact, PBMC stimulation with high dose of IFNλ1 showed variable mRNA levels for CXCL9, CXCL10 and CXCL11, suggesting that, among leukocytes, pDCs could be responsible for the production of these chemokines (155).

To date, most of the information about pDC responsiveness to IFNλs stimulation relies on pDCs identified among PBMCs by flow cytometry analysis, and are mainly based on IFNλ1 properties. Authors showed that IFNλ1 stimulation of PBMCs results in enhancement of surface CD80, CD83, ICOS-L, and MHC-I expression on pDCs (21,143). Moreover, expression of CD62L and CCR7 was increased in pDCs following PBMC-treatment with IFNλ1, providing evidence for a role of IFNλ1 in pDC maturation and trafficking. Finally, IFNλ1 has been shown to counteract the proapoptotic effect exerted by Dexamethasone (DEX) in pDCs as measured by Annexin V binding and expression of active caspase-3 (21), suggesting that type III IFNs can exert positive feedback to keep the pDCs alive at least over the short-run. The protective effect of type III IFNs for pDCs could be

important in the case of viral infection, preventing cells apoptosis and enhancing their functions during antiviral activity.

IFNλ-mediated stimulatory properties have been proved also on freshly isolated pDCs from blood of healthy donors. pDCs have been unequivocally shown to respond to IFNλs in terms of: i) MX1 mRNA induction by IFNλ3-stimulated pDCs (156); ii) increase in IFNα production after incubation with IFNλ1 plus CpG-A (134); iii) inhibition of IFNγ, IL-13, and IL-10 production by cocultures of IFNλ1-treated pDCs with allogenic T cells (143). As far as can be ascertained from the literature, no other information is available on pDC functional responses to IFNλs.

II. FIRST AIM OF THE STUDY

First aim of the study

TASK 1: CHARACTERIZATION OF PECULIAR pDC FUNCTIONS IN RESPONSE TO IFNλ3

Although the interplay between DCs and members of the IFNλ family is becoming increasingly relevant, particularly at the light of their key role in induction of the antiviral state and control of HCV replication (157), the immunomodulatory activities of IFNλs in pDCs are poorly defined.

pDCs constitute a nonparenchymal cell population that has been suggested to contribute to the intrahepatic IFN response during HCV infection, together with $CD141^+$ mDCs, which are enriched in the liver (158). $CD141^+$ mDCs and pDCs recognize HCV-infected hepatoma cells in a TLR3- and exosome-mediated fashion, respectively, and consequently produce IFNλs and IFNα (68,134). This mechanism needs to be still confirmed in the infected liver, even though it is conceivable that IFNα and IFNλ production by nonparenchymal cells could contribute to ISG induction by stimulating IFNαR and IFNλR (120,158).

IFNλ3 has been shown to inhibit HCV replication in three independent models, confirming its important role in the context of HCV pathogenesis (142). Moreover, single nucleotide polymorphisms (SNPs) detectable close to IFNλ3, but not close to IFNλ1 and IFNλ2 genes, have been defined as important predictors of HCV clearance by the infected host. Genetic variants within or close to IFNλ3 gene (such as rs12979860 and rs8099917 SNPs) are associated with spontaneous and treatment-induced outcome of HCV infection (159–161). However, the mechanisms by which IFNλ3 polymorphisms affect the efficacy of HCV clearance remain to be determined. All in all, data suggest that a comprehensive characterization of IFNλ3–mediated immunomodulatory activity on IFNλ-responsive cell populations, such as pDCs, need to be performed.

For this first part of my project, I characterized pDC responses to IFNλ3 stimulation, initially to confirm previous findings reported in literature for IFNλ1 on pDC survival and modulation of surface markers. Subsequently, I examined how

IFNλ3 treatment influences other pDC responses, such as gene expression induction and cytokine production.

2.1 MATERIALS AND METHODS

Cell isolation and culture conditions

PBMCs were isolated, under endotoxin-free conditions, from buffy coats of healthy donors after Ficoll-Hypaque gradient centrifugation (162). pDCs and $CD14^+$-monocytes were then isolated using, respectively, the BDCA-4 Diamond Isolation Kit and the Human Monocyte Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) (163), according to the manufacturer's instructions. After isolation, cells were suspended in RPMI 1640 medium supplemented with 10 % low-endotoxin fetal bovine serum (Sigma, Saint Louis, MO, USA) and either immediately analyzed for antigen expression, or cultured in 96-well tissue culture plates for functional assays. Purity of isolated pDCs (>98 %) and $CD14^+$-monocytes (>97 %) was determined by flow cytometry analysis (163). Our healthy donors were: i) all caucasians; ii), 18-65 years old; iii) periodically checked for blood exams; iv) 3:1 as a male:female ratio.

Cell stimulation

0.5×10^6 pDCs in 100 μl were usually plated in 96-well U-bottom plates (Costar, Corning Incorporated, Corning, NY), incubated in the presence or the absence of usually 30 ng/ml IFNλ3 (R&D, Minneapolis, MN, USA), 30 ng/ml IFNλ1 (R&D), 20 ng/ml IL-3 (Miltenyi) or their combination, 5 μM R837 (InvivoGen, San Diego, CA, USA), 100 U/ml IFNα (Pegasys®, Genetech, South San Francisco, CA, USA), 0.1-10 ng/ml TNFα (Peprotech) and then cultured at 37°C, 5% CO2 atmosphere for the times indicated. In all the experiments IL-3 was used at 20 ng/ml and IFNλ3 at 30 ng/ml, based on preliminary dose-response studies on gene expression induction, surface antigen modulation and cell viability. In selected experiments, pDCs were preincubated for 15 min with 5 μg/ml etanercept (a dimeric fusion protein that consists of the extracellular ligand-binding portion of the human 75 kDa TNF receptor linked to the Fc portion of the human IgG1, ENBREL®, Amgen, Thousand Oaks, CA, USA), 2.5 μg/ml adalimumab (a human-derived recombinant IgG1 monoclonal antibody, HUMIRA®, Abbott

Biotechnology, Illinois, USA), 2 μg/ml infliximab (a mouse/human chimeric IgG1 monoclonal antibody, REMICADE®, Horsham, PA, USA) or their isotype control Abs (human IgG1, from eBioscience, San Diego, CA, USA), as well as 5 μg/ml αIFNαR (PBL Interferon Source, Piscataway, NJ, USA) or its isotype control Abs (mouse IgG2a from R&D) before treatment. In other experiments, 0.125×10^6 $CD14^+$-monocytes in 50 μl were plated in 96-well flat-bottom plates in the presence or the absence of pDC-derived supernatants or 0.1 ng/ml TNFα. After 1 h, cells were collected and centrifuged at 400 × g for 5 min. Supernatants were harvested and immediately frozen at -80° C, while the corresponding cell pellets were either used for flow cytometry analysis or lysed for RNA extraction.

Table 1 summarize all the culture conditions, the stimuli and the various neutralizing antibodies that were used to specifically block the effects of the cytokine or membrane-bound receptors under investigation.

Table 1. List of culture conditions and neutralizing antibodies

Cell types	Stimuli	Neutralizing antibodies	Concentration
pDC	IFNλ3		30 ng/ml
	IFNλ1		30 ng/ml
	IL3		20 ng/ml
	R837		5 μM
	IFNα		100 U/ml
	TNFα		0.1-10 ng/ml
		etanercept	5 μg/ml
		adalimumab	2.5 μg/ml
		infliximab	2 μg/ml
		αIFNαR	5 μg/ml
$CD14^+$	TNFα		0.1 ng/ml
		etanercept	5 μg/ml
		adalimumab	2.5 μg/ml
		Spnt. Resting or IFNλ3-activated pDCs	

Flow cytometry analysis

Determination of antigen expression

To perform phenotypic studies (163), pDCs were first treated with 5 % human serum, and then stained for 20 min at room T, using the following mAbs: FITC anti-CD303 (Miltenyi), PE-Cy7 anti-CD123 (BioLegend, San Diego, California, USA), APC anti-human CD62L (Miltenyi) APC-Cy7 anti-HLA-DRα (BioLegend), PE anti-CD86 (BioLegend), anti-CD83 (Miltenyi) and their related isotype controls. For IFNλR1 detection, I used 2 μg/ml PE anti-IFNλR1 and, as isotype control Ab, PE mouse IgG2a (both from BioLegend), while IL10R2 expression was assessed by indirect staining using 10 μg/ml of an unconjugated mouse anti-IL-10R2 mAb [clone 4B2, kindly provided by Dr. K.W. Moore (when he was at DNAX institute in Palo Alto, CA, USA)], or its isotype control mAb (unconjugated mouse IgG1, from Biolegend), followed by an incubation with 4 μg/ml secondary PE goat anti-mouse pAbs (Biolegend) (164). Sample fluorescence was then measured by using an eight-color MACSQuant Analyzer (Miltenyi), data analysis performed by FlowJo software Version 8.8.6 (TreeStar).

Table 2 lists the fluorochrome-conjugated mAbs used in each staining.

Determination of apoptosis

Phenotypic cell analysis under the various experimental conditions was performed in live cells. For analysis of apoptosis, live cells were identified as singlet Vybrant^{TM} DyeCycle^{TM} Violet-negative cells (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions (163).

Table 2. List of fluorochrome-conjugated mAbs for FACS analysis

Fluorochrome-conjugated mAbs	Provider
CD303	Miltenyi
CD86	BioLegend
CD83	Miltenyi
IFNλR1	BioLegend
CD16	BioLegend
CD123	BioLegend
CD62L	Miltenyi
CD14	Miltenyi
HLA-DRα	Biolegend

Gene expression studies

Total RNA was extracted from pDCs and $CD14^+$-monocytes after lysis by RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer's instructions. To completely remove any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation. Purified total RNA was then reverse-transcribed into cDNA, as previously described (162). Gene expression studies were performed by reverse transcription real-time PCR (RT-qPCR), using gene-specific primer pairs (Life Technologies) available in the public database RTPrimerDB (http://medgen.ugent.be/rtprimerdb) under the following entry codes: TNFα (3551), CXCL10 (3537), IFNα (all genes) (3541), CXCL8 (3553), IFIT1 (3540), ISG15 (3547), RPL32 (8775), CCL4 (3535), IκBα (7888). Total RNA (usually extracted from 50000 pDCs or 125000 $CD14^+$-monocytes) was reverse transcribed by Superscript III (Life Technologies) while qPCR was carried out using Fast SYBR® Green Master Mix (Life Technologies). Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression (MNE) units after RPL32 normalization.

Measurement of soluble mediators

IFNα, CXCL10 and TNFα production was measured in pDC-derived supernatants using specific ELISA kits purchased from, respectively, Mabtech (Nacka Strand, Sweden)(IFNα, 7 pg /ml detection limit), R&D (CXCL10, 30 pg /ml detection limit) and eBioscience (TNFα, 4 pg /ml detection limit), according to the manufacturer's instructions.

Immunoblots

100000 pDCs were incubated with or without 30 ng/ml IFNλ3 for 45 and 90 min before blocking the stimulation in ice-cold PBS supplemented with 2 mM DFP and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$). Whole cell extracts were prepared and subjected to immunoblots by standard procedures (162) using 1:1.000 rabbit polyclonal Abs anti-phospho-STAT2 (Tyr689) (Millipore, Darmstadt, Germany), 1:1.000 anti-phospho-STAT1 (Tyr701) rabbit pAbs (Cell Signaling, Beverly, MA, USA), 1:500 anti-total-STAT1 or anti-total-STAT2 rabbit pAbs (both from Santa Cruz Biotechnology, Dallas, TX, USA). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis included one-way or two-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's post hoc test, respectively. Values of $P < 0.05$ were considered statistically significant. Statistical analysis was performed using Prism Version 6.0 software (GraphPad).

2 RESULTS (i)

2.2.1 RESULTS AND RELATED DISCUSSION ARE DESCRIBED IN THE FOLLOWING PUBLICATION:

Endogenously produced TNFα contributes to the expression of CXCL10/IP-10 in IFN-λ3-activated plasmacytoid dendritic cells

Results (i)

Epub ahead of print September 17, 2015 - doi:10.1189/jlb.3VMA0415-144R

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Article

Endogenously produced TNF-α contributes to the expression of CXCL10/IP-10 in IFN-λ3-activated plasmacytoid dendritic cells

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ABSTRACT

The interplay between IFN-λs and dendritic cells is becoming increasingly relevant, particularly in light of their key role in inducing the antiviral state, including in hepatitis C virus infection. In this work, we have analyzed extensively how human plasmacytoid dendritic cells respond to IFN-λ3. We report that plasmacytoid dendritic cells incubated with IFN-λ3 prolong their survival; alter their expression pattern of surface HLA-DRα, CD123, CD86, and CD303; and time dependently produce IFN-α, CXCL10/IFN-γ-induced protein 10, and even modest quantities of TNF-α. Nevertheless, endogenously produced TNF-α, but not IFN-α, was found to be essential for driving the expression of CXCL10/IFN-γ-induced protein 10 in IFN-λ3-treated plasmacytoid dendritic cells, as revealed by neutralizing experiments by use of adalimumab, etanercept, and infliximab. We also observed that based on the kinetics and levels of IFN-α and CXCL10/IFN-γ-induced protein 10 produced by their IFN-λ3-treated plasmacytoid dendritic cells, healthy donors could be categorized into 2 and 3 groups, respectively. In particular, we identified a group of donors whose plasmacytoid dendritic cells produced modest quantities of CXCL10/IFN-γ-induced protein 10; another one whose plasmacytoid dendritic cells produced elevated CXCL10/IFN-γ-induced protein 10 levels, already after 18 h, declining thereafter; and a 3rd group characterized by plasmacytoid dendritic cells releasing very high CXCL10/IFN-γ-induced protein 10 levels after 42 h only. Finally, we report that in plasmacytoid dendritic cells, equivalent concentrations of IFN-λ3 and IFN-λ1 promote survival, antigen modulation, and cytokine production in a comparable manner and without acting additively/synergistically. Altogether, data not only extend the knowledge on the biologic effects that IFN-λs exert on plasmacytoid dendritic cells but also add novel light to the networking between IFN-λs and plasmacytoid dendritic cells in fighting viral diseases. *J. Leukoc. Biol.* 99: 000–000; 2016.

Abbreviations: ADA = adalimumab, APC = allophycocyanin, CD62L = cluster of differentiation 62 ligand, DC = dendritic cell, DEX = dexamethasone, ETA = etanercept, HBV = hepatitis B virus, HCV = hepatitis C virus, ICOS-L = ICOS ligand, IFIT1 = IFN-induced protein with tetratricopeptide repeats 1, IP-10 = IFN-γ-induced protein 10, ISG = IFN-stimulated gene, MFI = mean fluorescence intensity, MHC-I/II = MHC class I/II, MNE = mean normalized expression, MX1 = myxovirus resistance 1, pDC = plasmacytoid dendritic cell, RPL32 = ribosomal protein L32, RT-qPCR = real-time quantitative PCR, SNP = single nucleotide polymorphism

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

IFNs are cytokines that are crucial for the establishment of innate and adaptive immune mechanisms aimed at destroying intracellular pathogens, particularly viruses [1]. Based on differences in their sequence, structure, receptor use, and biologic activities, IFNs are divided into 3 types: type I, mainly represented by IFN-α and IFN-β; type II, by IFN-γ; and type III, which includes the IFN-λ family, comprising IFN-λ1 or IL-29, IFN-λ2/IL-28A, IFN-λ3/IL-28B, and the more recently described IFN-λ4 [1, 2]. Although IFN-λs display structural similarities with both the type I IFNs and the IL-10 family of cytokines, IFN-λs and IFN-α share many biologic activities, in particular, direct antiviral effects [3]. Accordingly, antiviral activities of human IFN-λs have been demonstrated in cell cultures infected with influenza virus, HIV, HBV, and HCV [2–4]. IFN-λ3 has been shown to inhibit HCV replication in 3 independent HCV models [5]. Moreover, since the identification of SNPs, detectable near *IFN-λ3*, as important predictors of spontaneous or after-treatment HCV clearance [6–9], the role of IFN-λ3 in the context of HCV pathogenesis and progression seems particularly relevant [1, 2]. However, how IFN-λ3 polymorphisms translate into influencing the outcome of HCV disease has not been clarified yet.

IFN-λs, similarly to type I IFNs, signal through the JAK/STAT pathway, namely through STAT1 and STAT2, which ultimately induces sets of >300 ISGs that are important for their biologic activities [3, 4]. ISGs encode a variety of proteins, including ISG15, MX1, IFIT1, and CXCL10/IP-10, able, for instance, to inhibit viral replication, promote the degradation of viral nucleic acids, or modulate immune responses [10, 11]. Nonetheless, because of their different antiviral potency in some models, diverse induction patterns, and differential tissue expression of

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their corresponding receptor subunits, it is clear that type I and type III IFN antiviral actions do not merely duplicate but probably complement each other [2, 3, 12]. All IFN-λs signal through the same heterodimeric receptor complex composed of a unique IFN-λR1 (also known as IL-28Rα or cytokine receptor family 2 member 12) chain and IL-10R2 [3, 13]. Whereas IL-10R2 is ubiquitously expressed, IFN-λR1 displays a restricted tissue expression that is limited to epithelial cells of the respiratory, gastrointestinal, and reproductive tracts or to hepatocytes [14, 15]. Interestingly, in cells of the immune system, only pDCs and less prominently, B cells, express IFN-λR1 [16–18], but only pDCs have been unequivocally shown to respond to IFN-λs, in terms of altered CD80 expression [16], STAT1 phosphorylation activation [17], and MX1 mRNA induction [19].

DCs are cells that play a pivotal role at the interface between innate and adaptive immune responses [20]. In humans, DCs represent 0.3–0.5% of PBMCs and are typically grouped into 2 major subsets: conventional myeloid DCs and pDCs [20, 21]. Among DC subsets, pDCs are well recognized to produce massive amounts of type I IFNs and to acquire the capacity to present antigen upon exposure to viral stimuli [22, 23]. pDCs display a plasma cell morphology and, under steady-state conditions, carry low levels of MHC-I and -II and costimulatory molecules [24]. Peculiarly, pDCs strongly express the pattern recognition receptors TLR7 and TLR9 and are thus capable of recognizing ssRNA and unmethylated CpG-containing DNA ligands, respectively [25]. Importantly, pDCs regulate cell trafficking through the production of CXCL10/IP-10 and other chemokines [26, 27], provide help to NK cells [28], and also alter Th1/Th2 responses [29]. More recently, pDCs have also been shown to produce IFN-λ upon treatment with different types of viruses or coculture with HCV-infected cells or synthetic ligands for TLR7 and TLR9 [18, 30].

In this study, we have analyzed extensively how human pDCs respond upon incubation with IFN-λ3 and show that IFN-λ3-treated pDCs survive longer, undergo a partial maturation, and produce IFN-α, CXCL10/IP-10, and TNF-α. We also show that even though CXCL10/IP-10 totally depends on endogenously secreted TNF-α, donor-dependent factors likely condition, in a differential manner, the production of IFN-α and CXCL10/IP-10 by IFN-λ3-treated pDCs.

MATERIALS AND METHODS

Cell isolation and culture

PBMCs were isolated, under endotoxin-free conditions, from buffy coats of healthy donors after Ficoll-Hypaque gradient centrifugation [31]. pDCs and CD14+ monocytes were then isolated by use of, respectively, the BDCA-4 Diamond Isolation Kit and the Human Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) [32], according to the manufacturer's instructions. After isolation, cells were suspended in RPMI-1640 medium, supplemented with 10% low-endotoxin FBS (Sigma, St. Louis, MO, USA) and analyzed immediately for antigen expression or cultured in 96-well tissue-culture plates for functional assays. Purity of isolated pDCs (>98%; Supplemental Fig. 1A) and CD14+ monocytes was determined by flow cytometry analysis [32]. Our healthy donors were the following: 1) all caucasians; 2), 18–65 y old; 3) periodically checked for blood exams; 4) 3:1 as a male:female ratio.

Cell stimulation

pDCs (0.5×10^5) in 100 μl were usually plated in 96-well U-bottom plates (Costar; Corning, Corning, NY, USA), incubated in the presence or absence of usually 30 ng/ml IFN-λ3 (R&D Systems, Minneapolis, MN, USA), 30 ng/ml IFN-λ1 (R&D Systems), 20 ng/ml IL-3 (Miltenyi Biotec), 5 μM R837 (InvivoGen, San Diego, CA, USA), 100 U/ml IFN-α (Pegasys; Genentech, South San Francisco, CA, USA), and 0.1–10 ng/ml TNF-α (PeproTech, Rocky Hill, NJ, USA) and then cultured at 37°C, 5% CO_2 atmosphere, for the times indicated. In selected experiments, pDCs were preincubated for 15 min with 5 μg/ml ETA (a dimeric fusion protein that consists of the extracellular ligand-binding portion of the human 75 kDa TNFR linked to the Fc portion of the human IgG_1, ENBREL; Amgen, Thousand Oaks, CA, USA), 2.5 μg/ml ADA (a human-derived $rIgG_1$ mAb, HUMIRA; Abbott Laboratories, Abbott Park, IL, USA), 2 μg/ml infliximab (a mouse/human chimeric IgG_1 mAb, REMICADE; Janssen Biotech, Horsham, PA, USA), or their isotype control antibodies (human IgG_1; eBioscience, San Diego, CA, USA), as well as 5 μg/ml αIFNAR (PBL InterferonSource, Piscataway, NJ, USA) or their isotype control antibodies (mouse IgG_{2a}; R&D Systems), before treatment. In other experiments, 0.125×10^6 CD14+ monocytes in 50 μl were plated in 96-well flat-bottom plates in the presence or absence of pDC-derived supernatants or 0.1 ng/ml TNF-α. After 1 h, cells were collected and centrifuged at 400 *g* for 5 min. Supernatants were harvested and frozen immediately at −80°C, while the corresponding cell pellets were used for flow cytometry analysis or lysed for RNA extraction.

Flow cytometry analysis

To perform phenotypic studies [32], pDCs were first treated with 5% human serum and then stained for 20 min at room temperature by use of the following mAbs: FITC anti-CD303 (Miltenyi Biotec), PE-Cy7 anti-CD123 (BioLegend, San Diego, CA, USA), APC anti-human CD62L (Miltenyi Biotec), APC-Cy7 anti-HLA-DRα (BioLegend), PE anti-CD86 (BioLegend), anti-CD83 (Miltenyi Biotec), and their related isotype controls. For IFN-λR1 detection, we used 2 μg/ml PE anti-IFN-λR1 and as isotype control antibody, PE mouse IgG2a (both from BioLegend), whereas IL-10R2 expression was assessed by indirect staining by use of 10 μg/ml of an unconjugated mouse anti-IL-10R2 mAb [clone 4B2; kindly provided by Dr. K. W. Moore (when affiliated with DNAX Institute, Palo Alto, CA, USA)] or its isotype control mAb (unconjugated mouse IgG_1; BioLegend), followed by an incubation with 4 μg/ml secondary PE goat anti-mouse polyclonal antibodies (BioLegend) [33]. Sample fluorescence was then measured by use of an 8-color MACSQuant analyzer (Miltenyi Biotec) and data analysis performed by FlowJo software, version 8.8.6 (Tree Star, Ashland, OR, USA). Phenotypic cell analysis under the various experimental conditions was performed in live cells, identified as singlet Vybrant DyeCycle Violet-negative cells (Life Technologies, Carlsbad, CA, USA) the overall gating strategy for live cells is depicted in Supplemental Fig. 1A [32].

Gene-expression studies

Total RNA was extracted from pDCs and CD14+ monocytes after lysis by the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer's instructions. To remove completely any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation. Purified total RNA was then reverse transcribed into cDNA, as described previously [31]. Gene-expression studies were performed by RT-qPCR by use of gene-specific primer pairs (Life Technologies), available in the public database RTPrimerDB (http://www.rtprimerdb.org) under the following entry codes: TNF-α (3551), CXCL10 (3537), IFN-α (all genes; 3541), CXCL8 (3553), IFIT1 (3540), ISG15 (3547), RPL32 (8775), CCL4 (3535), and IκBα (7888). Total RNA (usually extracted from 50,000 pDCs or 125,000 CD14+ monocytes) was reverse transcribed by Superscript III (Life Technologies), whereas qPCR was carried out by use of Fast SYBR Green Master Mix (Life Technologies). Data were calculated by qGENE software (http://www.gene-quantification.de/download.html) and expressed as MNE units after RPL32 normalization.

Cytokine measurement

IFN-α, CXCL10/IP-10, and TNF-α production was measured in pDC-derived supernatants by use of specific ELISA kits, purchased from, respectively,

Mabtech (Nacka Strand, Sweden; IFN-α, 7 pg/ml detection limit), R&D Systems (CXCL10/IP-10, 30 pg/ml detection limit), and eBioscience (TNF-α, 4 pg/ml detection limit), according to the manufacturers' instructions.

Immunoblots

pDCs (100,000) were incubated with or without 30 ng/ml IFN-λ3 for 45 and 90 min before blocking the stimulation in ice-cold PBS, supplemented with 2 mM diisopropylfluorophosphate and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$). Whole-cell extracts were prepared and subjected to immunoblots by standard procedures [31] by use of 1:1000 rabbit polyclonal antibody anti-phospho-STAT2 (Tyr689; Millipore, Darmstadt, Germany), 1:1000 anti-phospho-STAT1 (Tyr701) rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), and 1:500 anti-total-STAT1 or anti-total-STAT2 rabbit polyclonal antibodies (both from Santa Cruz Biotechnology, Dallas, TX, USA). Blotted proteins were detected and quantified by use of the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis included 1-way or 2-way ANOVA, followed by Tukey's or Bonferroni's post hoc test, respectively. Values of $P < 0.05$ were considered statistically significant. Statistical analysis was performed by use of Prism, version 6.0, software (GraphPad Software, La Jolla, CA, USA).

RESULTS

IFN-λ3 promotes survival and antigen modulation in human pDCs

Initial experiments confirmed that pDCs, freshly isolated from the peripheral blood of healthy donors, display both subunits composing the IFN-λR, namely IFN-λR1 and IL-10R2 (Supplemental Fig. 1B) [17, 18, 34], as well as tyrosine phosphorylated STAT1 and STAT2 if incubated with IFN-λ3 (Supplemental Fig. 1C). In these latter, as well as in all subsequent, experiments, IFN-λ3 was used at 30 ng/ml. This was based on preliminary dose-response studies on gene-expression induction (Supplemental Fig. 2A), surface antigen modulation (Supplemental Fig. 2B), and survival (Supplemental Fig. 2C), which identified such concentration as the optimal one to evaluate the effects of IFN-λ3 in pDCs, in line with other studies [16–19].

Subsequent experiments revealed that IFN-λ3 maintains pDC survival for up to 42 h (**Fig. 1A** and **B**), as measured by the Vybrant DyeCycle Violet stain (Fig. 1C, showing a representative experiment). Notably, the positive effect of IFN-λ3 on pDC survival was found to be substantially comparable with that induced by IL-3 (Fig. 1A and B), a growth factor known to

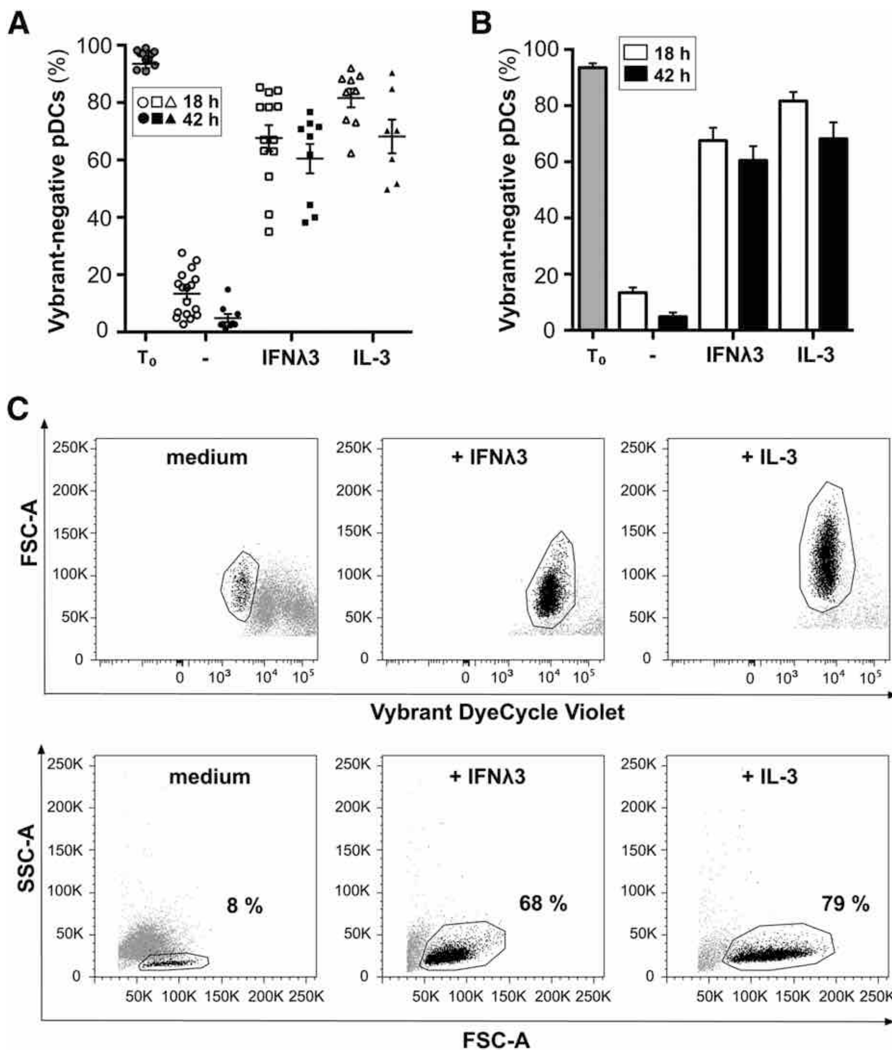

Figure 1. IFN-λ3 prolongs the survival of pDCs. pDCs were incubated with or without 30 ng/ml IFN-λ3 or 20 ng/ml IL-3 right after isolation from the blood. Cells were harvested at the 18 and 42 h time points and then stained by the Vybrant DyeCycle to assess their viability compared with freshly isolated pDCs (T_0) by flow cytometry analysis. (A) Results of all individual experiments in terms of viable cells for the conditions displayed (n = 8–16); (B) related means ± SEM. (C) Gating strategy used to identify pDCs incubated for 42 h that are live, from a representative experiment. (Upper) Viable pDCs gated as Vybrant DyeCycle-negative cells; (lower) cellular morphology and percentages. Gray and black dots stand for dead and live cells, respectively. The overall gating strategy is depicted in Supplemental Fig. 1. FSC-A, Forward-scatter-area; SSC-A, side-scatter-area.

maintain pDC viability [24]. Moreover, flow cytometric analysis confirmed [35, 36] that pDCs cultured for up to 42 h in medium only express levels of HLA-DRα (**Fig. 2A**), CD123 (Fig. 2B), CD83 (Fig. 2C), and CD86 (Fig. 2D), substantially similar to those observed in freshly isolated cells, whereas they significantly decrease CD303 ($P < 0.01$; Fig. 2E) and increase CD62L (in the latter case, at 42 h only; $P < 0.01$; Fig. 2F) levels. As compared with untreated cells, expression of HLA-DRα (Fig. 2A), CD123 (Fig. 2B), CD83 (Fig. 2C), and CD86 (Fig. 2D) in IFN-λ3-treated cells was up-regulated significantly (for CD83 and CD86 only at 18 and 42 h, respectively, of culture), whereas that of CD62L and CD303 was down-regulated significantly at 18 and 42 h, respectively (Fig. 2E; see also representative plots in Supplemental Fig. 3). Such IFN-λ3-mediated pDC antigen modulation, again, substantially resembled that exerted by IL-3 (Fig. 2 and Supplemental Fig. 3) [24, 37], with some exceptions: IL-3, in fact, was found to be significantly more potent than IFN-λ3, either in up-regulating expression of HLA-DRα at 42 h (Fig. 2A) and of CD86 at 18 h (Fig. 2D) or in down-regulating CD303 (Fig. 2E) and CD62L (Fig. 2F) expression at 18 and 42 h, respectively. On the other hand, IL-3 was significantly less efficient than IFN-λ3 in up-regulating CD83 after 18 h (Fig. 2C).

Taken together, data demonstrate that IFN-λ3 potently acts on pDCs in terms of enhanced survival and modulation of surface markers. Data also indicate that at least phenotypically [35, 38], IFN-λ3 induces a partial maturation of pDCs.

IFN-λ3 induces the production of IFN-α and CXCL10/IP-10 by human pDCs

We then evaluated whether, in pDCs, IFN-λ3 could induce the production of IFN-α and in turn, CXCL10/IP-10, as this T cell attractant chemokine, under a variety of conditions [39–41], depends on endogenously released type I IFN. No CXCL10/IP-10 and IFN-α were measurable in supernatants from pDCs cultured for up to 42 h in medium only (**Fig. 3**). We instead observed that IFN-λ3-treated pDCs produce and release significant amounts of IFN-α (608 ± 159 pg/ml, $n = 16$; Fig. 3A) or CXCL10/IP-10 (764 ± 187 pg/ml, $n = 16$; Fig. 3B) after 42 h of incubation. However, only CXCL10/IP-10 could be measured at

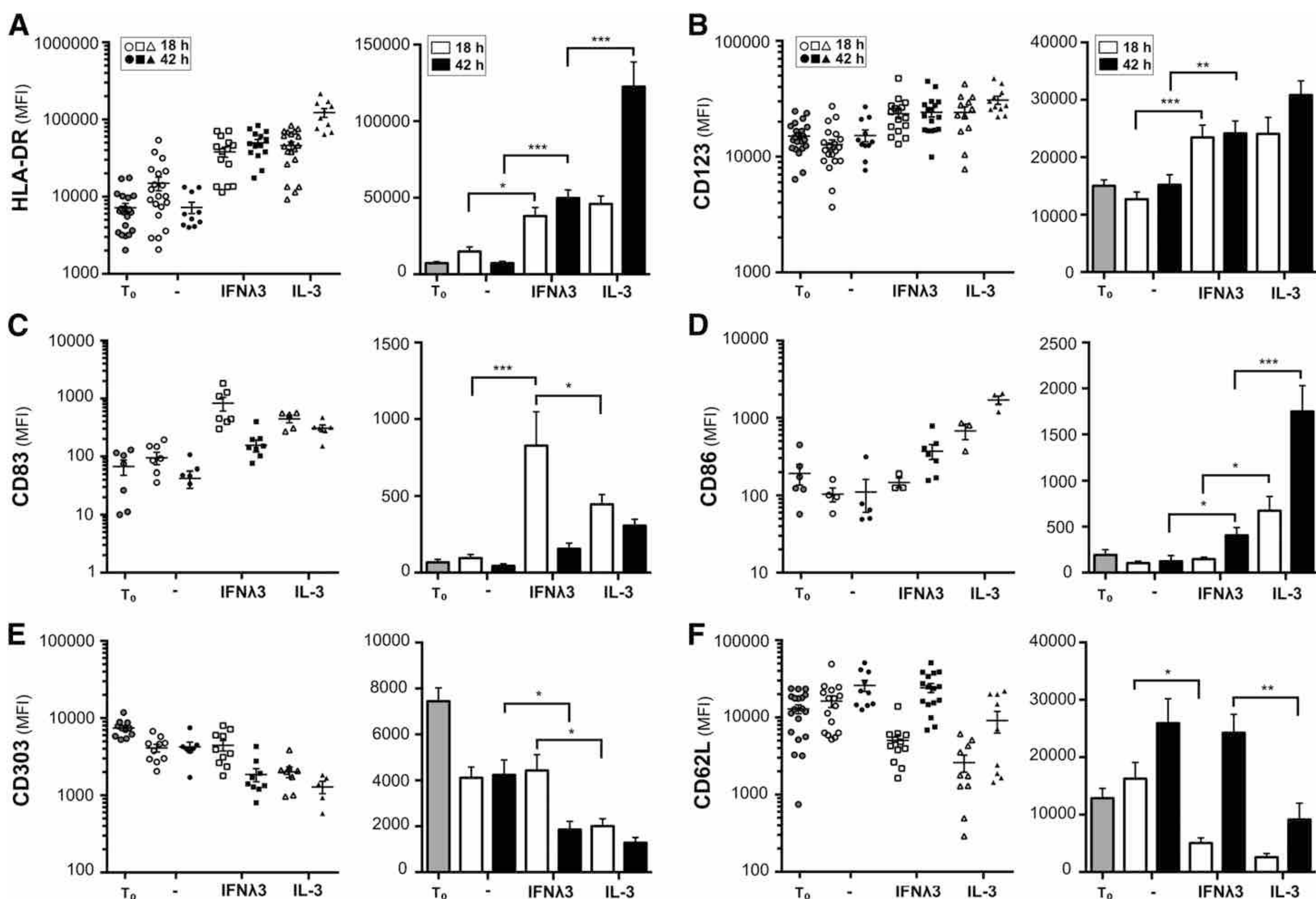

Figure 2. IFN-λ3 modulates the expression of several antigens in pDCs. After isolation, pDCs were incubated with or without 30 ng/ml IFN-λ3 or 20 ng/ml IL-3. At the 18 and 42 h time points, cells were harvested and analyzed for HLA-DR (A), CD123 (B), CD83 (C), CD86 (D), CD303 (E), and CD62L (F) expression by flow cytometry. For each antigen, the panels on the left report the results (as MFI) on a logarithmic scale of all individual measurements, whereas bar graphs on the right report their means ± SEM ($n = 5–20$) on a linear scale. MFI was calculated after subtracting the MFI given by the correspondent isotype control antibodies or the basal fluorescence. Significant variations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

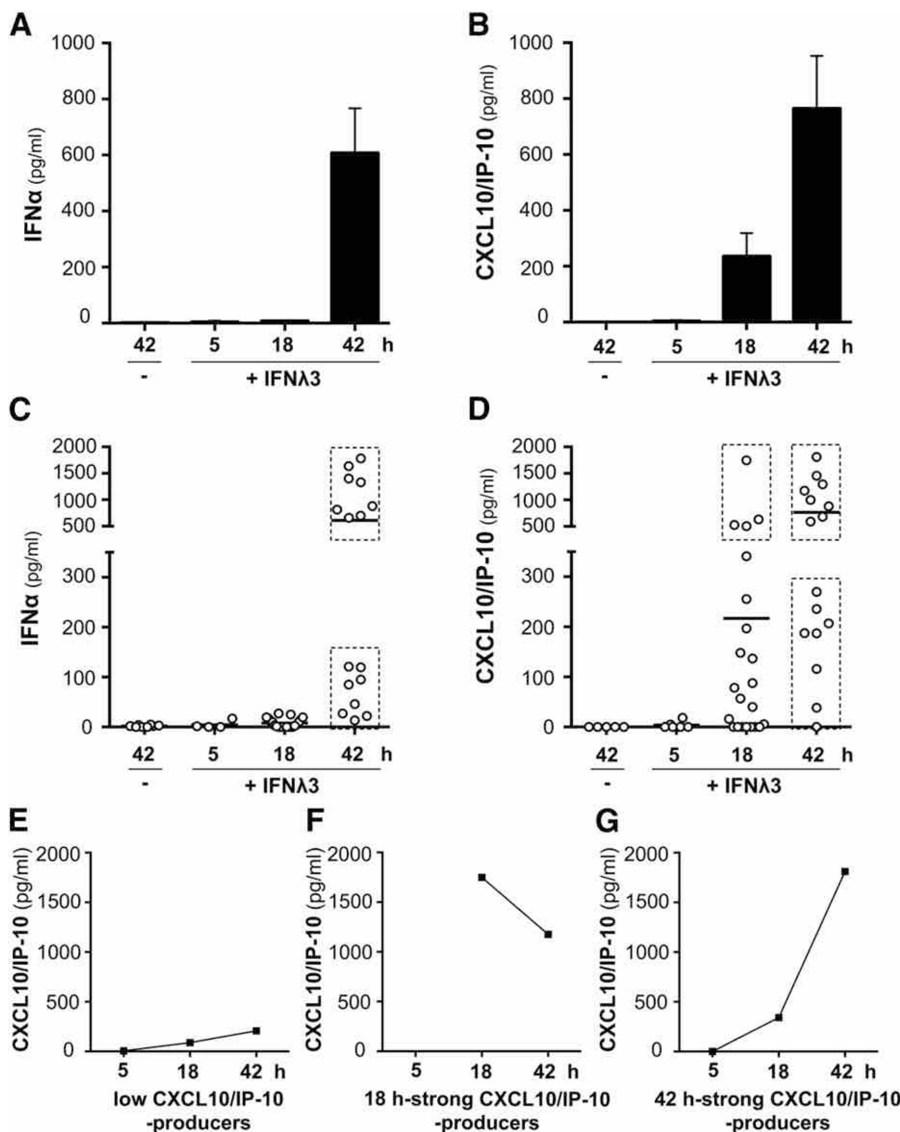

Figure 3. IFN-λ3 induces a time-dependent production of IFN-α and CXCL10/IP-10 by pDCs. After isolation, pDCs were incubated with or without 30 ng/ml IFN-λ3 for 5, 18, and 42 h. Cell-free supernatants were then collected and extracellular IFN-α (A and C) or CXCL10/IP-10 (B, D, and E–G) measured by ELISA. (A and B) Means ± SEM of all experiments (n = 13–18); (C and D) results of all individual experiments. pDCs incubated for up to 42 h in the absence of IFN-λ3 produced neither IFN-α nor CXCL10/IP-10. (E–G) Three different, reproducible patterns of CXCL10/IP-10 production by pDCs incubated with IFN-λ3.

remarkable levels after 18 h (216 ± 83 pg/ml, n = 22), suggesting that its expression precedes that of IFN-α (8 ± 3 pg/ml/18 h, n = 17). Accordingly, in most samples of pDCs incubated with IFN-λ3, an induction of CXCL10, but not IFN-α, mRNA could be detected as early as after 5 h, whereas an evident, although variable, accumulation of IFN-α and CXCL10 transcripts was present at 18 h (data not shown).

Although the graphical representations displayed in Fig. 3A and B (reporting the means ± SEM of IFN-α and CXCL10/IP-10 release calculated from all samples) would suggest that maximal production of IFN-α and CXCL10/IP-10 by pDCs would occur after 42 h incubation with IFN-λ3, that was not always the case for CXCL10/IP-10. In fact, we observed a very large variability in the levels of extracellular IFN-α (Fig. 3C) and CXCL10/IP-10 (Fig. 3D) measured in supernatants harvested from pDCs treated with IFN-λ3 (ranging from a few up to thousands picograms/milliliter), which, at least in some samples for CXCL10/IP-10, already reached their maximum at 18 h (Fig. 3D). More interestingly, we could retrospectively identify 3 reproducible patterns of CXCL10/IP-10 production by pDCs incubated with IFN-λ3 (Fig. 3D; dashed boxes), as better illustrated in representative experiments shown in Fig. 3E–G and globally summarized in **Fig. 4A–C**: a first group, herein referred to donors defined as “low CXCL10/IP-10 producers,” characterized by a modest production of CXCL10/IP-10 at 18 h (22 ± 11 pg/ml, n = 10), which even if remaining substantially low, significantly increases at 42 h (163 ± 24 pg/ml, n = 7; Figs. 3E and 4A); a second group, herein referred to donors defined as “18 h strong CXCL10/IP-10 producers,” characterized by remarkably elevated levels of CXCL10/IP-10 production already after 18 h (865 ± 297 pg/ml, n = 4), which do not further increase at 42 h (722 pg/ml, n = 2; Figs. 3F and 4B); and finally, a 3rd group, herein referred to donors defined as “42 h strong CXCL10/IP-10 producers,” characterized by very high CXCL10/IP-10 levels detectable after 42 h (1320 ± 264 pg/ml, n = 7; Figs. 3G and 4C).

Similarly to CXCL10/IP-10, 2 patterns of IFN-α production by IFN-λ3-treated cells were also reproducibly distinguishable based on the extracellular cytokine levels measured at the 42 h time

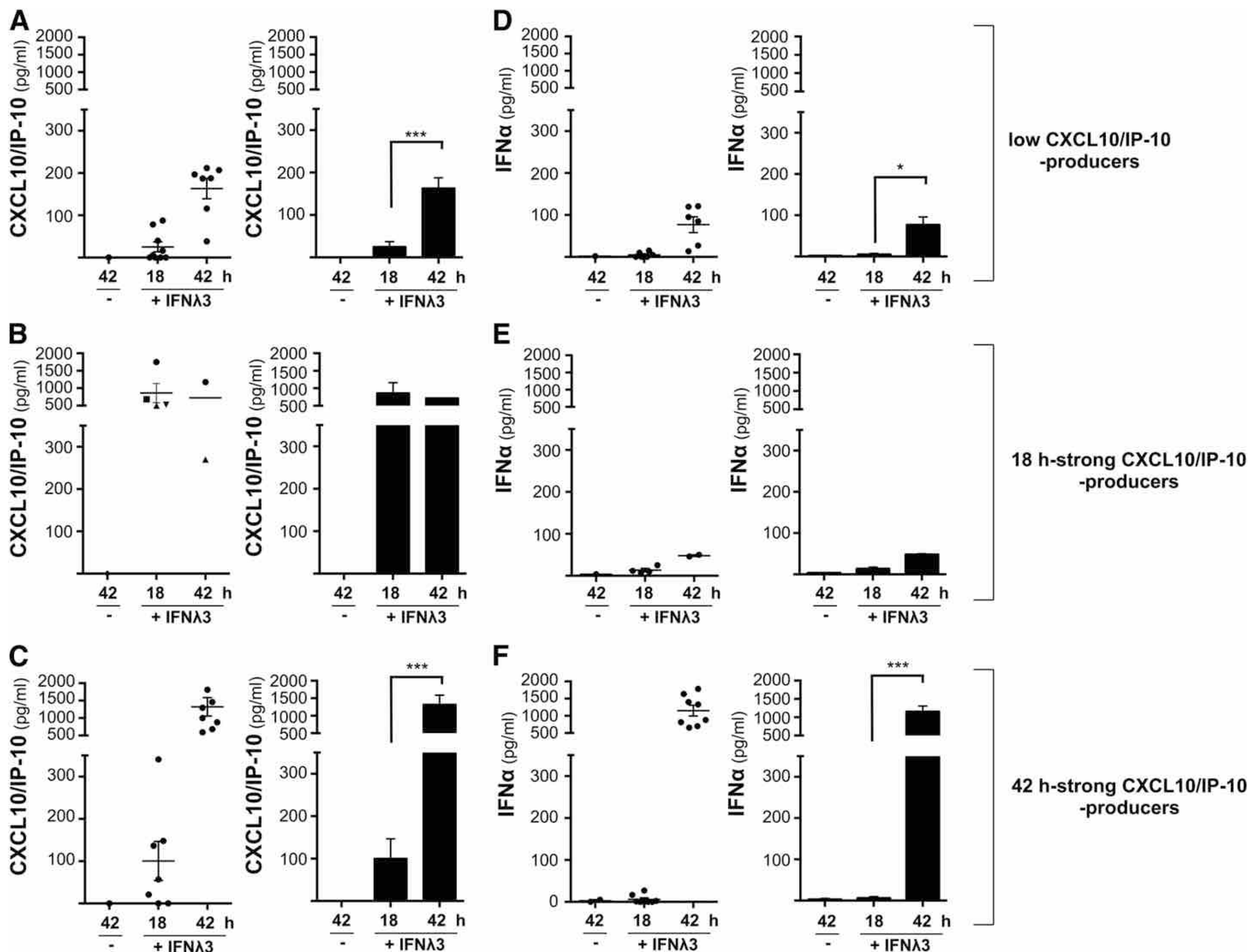

Figure 4. Relationship between the levels of CXCL10/IP-10 and the levels of IFN-α induced by IFN-λ3 in pDCs. (A and D) Extracellular production of CXCL10/IP-10 and IFN-α, respectively, measured in pDC-derived supernatants harvested from all low CXCL10/IP-10 producers. (B and E) Extracellular production of CXCL10/IP-10 and IFN-α, respectively, from the 18 h strong CXCL10/IP-10 producers. (C and F) Extracellular production of CXCL10/IP-10 and IFN-α, respectively, from the 42 h strong CXCL10/IP-10 producers. For each group, left panels report the absolute values of CXCL10/IP-10 (A–C) and IFN-α (D–F) production by IFN-λ3-treated pDCs (from all individual experiments); while the right panels (bar graphs) display the means ± SEM of the values reported in the left ones. pDCs incubated in the absence of IFN-λ3 for up to 42 h produced neither IFN-α nor CXCL10/IP-10. Please note that in B, each single experiment is identified by the same symbol. Significant increases: *$P < 0.05$, ***$P < 0.001$.

point (Fig. 3C): 1 of them displaying IFN-α amounts <150 pg/ml (Fig. 3C, upper dashed box; here defined as "low IFN-α producers") and the other 1 >500 pg/ml (Fig. 3C, lower dashed box; here defined as "strong IFN-α producers"). Interestingly, by matching the amounts of IFN-α and CXCL10/IP-10, measured in the same samples (Fig. 4), it seemed evident that the donors whose pDCs produced low levels of IFN-α (Fig. 4D) mainly corresponded to the low CXCL10/IP-10 producers (Fig. 4A), a few of them (Fig. 4E) coinciding with the 18 h strong CXCL10/IP-10 producers (Fig. 4B), whereas the donors whose pDCs produced very high levels of IFN-α (Fig. 4F) all corresponded to the 42 h strong CXCL10/IP-10 producers (Fig. 4A). Such a correspondence was corroborated by calculating the Pearson correlation coefficient, which proved that the release of CXCL10/IP-10 and IFN-α by IFN-λ3-treated pDCs was statistically correlated after 42 ($r = 0.683$, $P < 0.01$) but not after 18 ($r = 0.219$, $P = 0.313$) h of incubation. Furthermore, the percentage of live (Vybrant-negative) pDCs after 18 or 42 h of incubation with IFN-λ3 was found to be substantially similar within the 3 CXCL10/IP-10 (**Fig. 5A**) or the 2 IFN-α- (Fig. 5B) producer groups, indicating that the variable CXCL10/IP-10 and IFN-α production was not related to differences in pDC viability/death.

Taken together, data not only prove that pDCs treated with IFN-λ3 produce and release significant quantities of IFN-α and CXCL10/IP-10, but also uncover that the extent of IFN-α and CXCL10/IP-10 production by IFN-λ3-treated pDCs is very variable and likely influenced by donor-dependent factors.

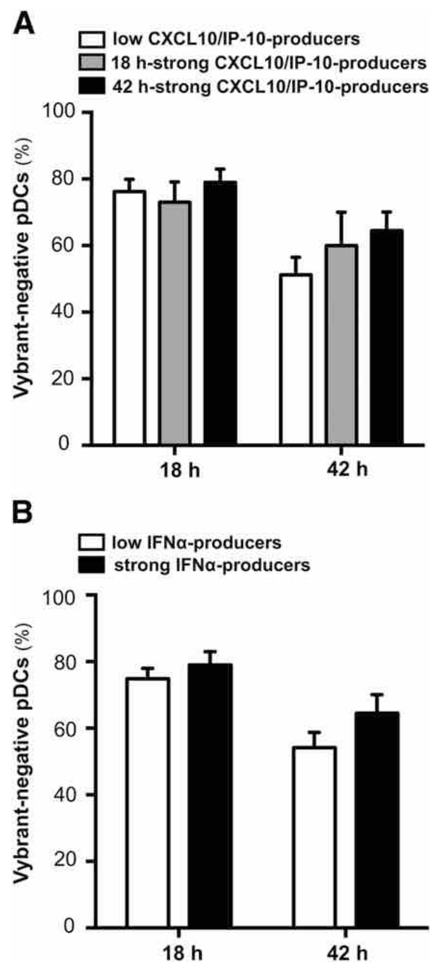

Figure 5. Survival of pDCs treated with IFN-λ3 categorized according to the CXCL10/IP-10 or IFN-α producer groups. Viability of pDCs after 18 and 42 h of incubation with 30 ng/ml IFN-λ3, as analyzed by flow cytometry. The percentage of live (Vybrant-negative) pDCs within the 3 CXCL10/IP-10 producer groups is shown in (A), while that within the 2 IFN-α producer groups is shown in (B) (means ± SEM; n = 3–4).

Endogenous IFN-α is only partially required to induce CXCL10/IP-10 in IFN-λ3-treated pDCs

To ascertain definitively whether the induction of CXCL10/IP-10 expression in IFN-λ3-treated pDCs depends on endogenous IFN-α, we performed experiments in which pDCs were pre-treated with antibodies neutralizing the IFN-αR [42] before their incubation with IFN-λ3 (**Fig. 6**). Luckily, the donors used in these experiments happened to fall, by chance, into the 42 h strong CXCL10/IP-10 producer group. In these experiments, αIFN-αR antibodies only modestly inhibited the induction of CXCL10 mRNA at 18 h (Fig. 6A) or the production of CXCL10/IP-10 at 42 h (Fig. 6B), whereas they completely blocked the induction of CXCL10/IP-10 production and mRNA expression by pDCs incubated with IFN-α (Fig. 6D and E). Under the same experimental conditions, isotype control antibodies did not affect CXCL10/IP-10 production induced by IFN-λ3 and IFN-α (Fig. 6C and F). Taken together, data demonstrate that CXCL10/IP-10 produced by pDCs after 42 h of incubation with IFN-λ3 is only partially controlled by endogenous IFN-α.

Endogenous TNF-α is crucial for the induction of CXCL10/IP-10 in IFN-λ3-treated pDCs

It has been demonstrated that under several conditions [43–45], transcription of CXCL10 can be cooperatively induced by STAT1-activating stimuli (such as type I and type II IFNs), acting in combination with NF-κB-activating agonists, including TNF-α. Human pDCs are known to produce TNF-α, for instance in response to R837 (Imiquimod) [46, 47]. Therefore, we investigated whether IFN-λ3 could trigger the production of TNF-α in pDCs and if so, whether TNF-α could endogenously play a role in activating CXCL10/IP-10 expression. As shown in **Fig. 7A** and **B**, very low but detectable amounts of TNF-α could be measured in supernatants harvested from untreated and IFN-λ3-treated pDCs, in the latter case, slowly increasing after 18 h and reaching significant levels up to 42 h. Concomitantly, TNF-α mRNA accumulation significantly increased at 18 h in IFN-λ3-treated pDCs (Fig. 7C). Notably, the yields of TNF-α were not as variable as the yields of IFN-α and CXCL10/IP-10 measured in the same supernatants, as also confirmed by the coefficient of variability analysis (data not shown) [48]. Despite of these low TNF-α amounts, pDC incubation in the presence of TNF-α blockers, including ETA, ADA, or infliximab [49], completely prevented the induction of CXCL10 mRNA at 18 h (Fig. 7D, and data not shown), as well as of CXCL10/IP-10 production at 18 and 42 h (Fig. 7E, and data not shown), in response to IFN-λ3, regardless of the "CXCL10/IP-10 producer" group. Under the same experimental conditions, isotype control antibodies did not affect, in pDCs treated with IFN-λ3, either the production of CXCL10/IP-10 or the modulation of membrane markers (data not shown). On the other hand, ETA inhibited neither the survival of pDCs incubated with IFN-λ3 for 42 h (Supplemental Fig. 4A) nor the production of CXCL10/IP-10 induced by R837 in pDCs (Supplemental Fig. 4B), whereas both ETA and ADA completely suppressed the induction of CXCL8 mRNA triggered by 10 ng/ml TNF-α in pDCs (Supplemental Fig. 4C). Surprisingly, TNF-α alone, at doses ranging from 0.01 to 10 ng/ml, was found unable or able to trigger only minute amounts of CXCL10/IP-10 by pDCs cultured for up to 42 h (data not shown). However, supernatants harvested from pDCs, incubated for 18 h with IFN-λ3, but not with medium only, once transferred to $CD14^+$ monocytes for 1 h in the presence or absence of TNF-α inhibitors, induced a TNF-α-dependent CCL4 (**Fig. 8A**) and IκBα (Fig. 8B) mRNA expression. Importantly, neither was the amounts of TNF-α contained in these supernatants (~15 pg/ml) further increased once added to $CD14^+$ monocytes nor was TNF-α mRNA induced by them (data not shown), proving that the biologic effects mediated by pDC-derived supernatants were promoted by the TNF-α exclusively derived from IFN-λ3-treated pDCs. Moreover, whereas addition of 0.1 ng/ml TNF-α to $CD14^+$ monocytes induced CCL4 (Fig. 8C) and IκBα (Fig. 8D) mRNA expression, addition of IFN-λ3 neither did so nor potentiated the action of TNF-α (Fig. 8C and D), confirming that monocytes do not respond to IFN-λ3. Taken together, data demonstrate that IFN-λ3 induces the production of biologically active TNF-α by pDCs. Data also prove that endogenously produced TNF-α is essential for the induction of CXCL10/IP-10 in IFN-λ3-treated pDCs.

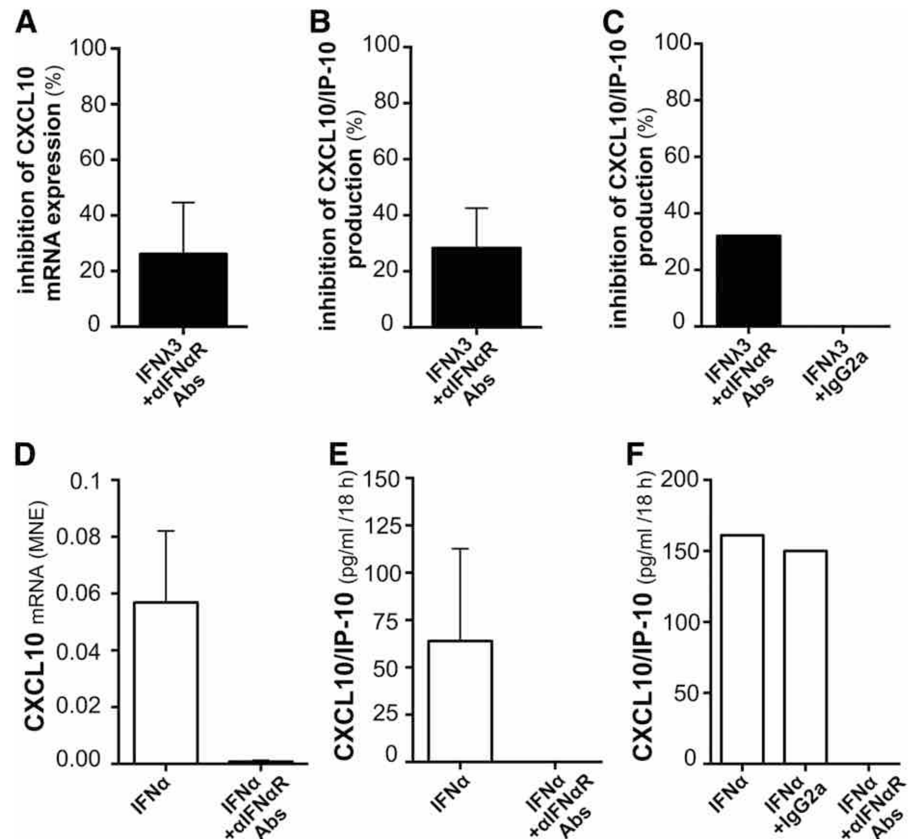

Figure 6. Role of endogenous IFN-α in mediating the induction of CXCL10/IP-10 in IFN-λ3-treated pDCs. pDCs were pretreated for 30 min with or without 5 μg/ml αIFN-αR or mouse IgG_{2a} (isotype control antibodies) and then incubated with IFN-λ3 (A and B) for 18 and 42 h or 100 U/ml IFN-α (C and D) for 5 and 18 h to perform CXCL10 gene-expression and protein-production studies. Bar graphs show the percentage of inhibition on both CXCL10 mRNA expression (A and D; means ± SEM; $n = 4$,) and CXCL10/IP-10 production (B and E; $n = 3$) exerted by αIFN-αR antibodies. Under the same experimental conditions, isotype control antibodies did not affect CXCL10/IP-10 production or mRNA expression induced by IFN-λ3 or IFN-α, as illustrated by C and F, displaying 1 of the experiments reported in B and E. Gene-expression data are depicted as MNE units after RPL32 mRNA normalization.

IFN-λ1 and IFN-λ3 are equally effective in promoting survival, antigen modulation, and cytokine production in pDCs

In a final series of experiments, we also addressed the potency of IFN-λ3, relative to that of IFN-λ1, on pDC survival and/or antigen modulation, as IFN-λ1 has been shown to counteract the proapoptotic effect exerted in pDCs by DEX [17], as well as to enhance their CCR7, CD62L, CD80, CD83, ICOS-L, and MHC-I expression levels [16, 17]. pDCs were cultured with or without 30 or 100 ng/ml IFN-λ1 in the presence or absence of 30 ng/ml IFN-λ3 (purchased from the same company). As shown in **Fig. 9**, the modulatory effects by 30 ng/ml IFN-λ3 or 30 ng/ml IFN-λ1 on pDC viability (Fig. 9A), as well as on pDC expression of CD86, CD83, and HLA-DRα (Fig. 9B), were found to be substantially similar. Furthermore, a combination of the 2 IFN-λ types did not provoke any additive/synergistic effects either (Fig. 9A and B). Maximal effects on both cell viability (Fig. 9A) and antigen levels (Fig. 9B) did not significantly change if IFN-λ1 were used at 100 ng/ml, consistent with the data obtained with IFN-λ3 (Supplemental Fig. 2).

We also measured the levels of CXCL10/IP-10, IFN-α, and TNF-α in supernatants from the pDCs used for these experiments, which retrospectively involved 3 donors belonging to the 42 h strong CXCL10/IP-10 producers. As shown in Fig. 9C, production of CXCL10/IP-10, IFN-α, and TNF-α by pDCs incubated for 42 h with 30 ng/ml IFN-λ1 was not significantly different from that triggered by 30 ng/ml IFN-λ3. Once again, a combination of IFN-λ1 and IFN-λ3 did not trigger any additive/synergistic cytokine production (Fig. 9C). Worthy of note is that in this group of experiments, the 42 h strong CXCL10/IP-10 producer pattern was reproduced also in response to IFN-λ1, further corroborating the validity of our observations.

DISCUSSION

Although the interplay between DCs and members of the IFN-λ family is becoming increasingly relevant, particularly at the light of their key role in induction of the antiviral state and, for instance, control of HCV replication [50, 51], the immunomodulatory activities of IFN-λs on pDCs are poorly defined. In this work, we report that human pDCs respond to IFN-λ3 in terms of enhanced survival, modulation of surface markers, gene expression induction, and cytokine production. In particular, we show that treatment of pDCs with IFN-λ3 promotes the following: 1) a maintenance of pDC viability at levels comparable with IL-3, 1 of the major survival factor for pDCs [24, 37]; 2) a change in the expression pattern of surface HLA-DRα, CD123, CD86, and CD303, consistent with a "partial" pDC maturation [35, 38, 46]; 3) an induction of typical ISG mRNAs, including IFIT1, ISG15, and CXCL10; 4) a time-dependent production of IFN-α, CXCL10/IP-10, and unexpectedly, also TNF-α (in modest amounts). These data not only extend previous observations, demonstrating that pDCs up-regulate MX1 mRNA upon incubation with IFN-λ3 [19], but also confirm that pDCs respond to IFN-λ family members, as reported previously for IFN-λ1. The latter IFN-λ type, in fact, has been shown to counteract the

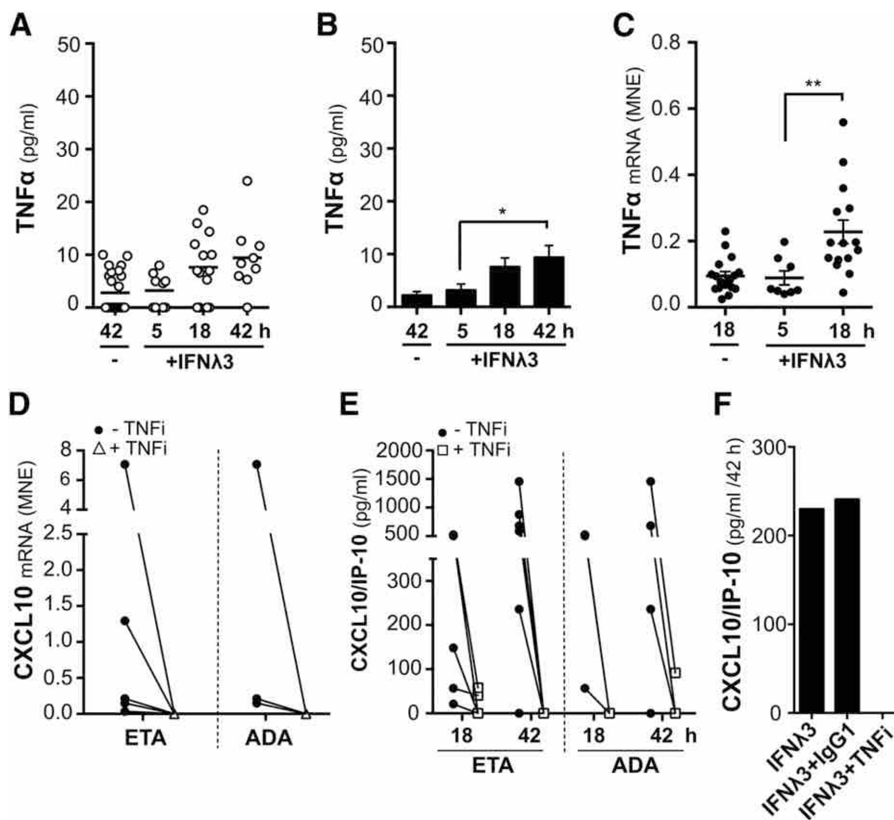

Figure 7. Role of endogenous TNF-α in mediating the induction of CXCL10/IP-10 in IFN-λ3-treated pDCs. pDCs were incubated with or without 30 ng/ml IFN-λ3 for up to 42 h to analyze gene expression and extracellular production of TNF-α. (A) Results of all individual measurements; (B) means ± SEM of A (n = 13–19). (C) TNF-α mRNA expression in IFN-λ3-treated pDCs. (D and E) pDCs were pretreated for 30 min with or without TNF-α inhibitors (TNFi), namely 5 μg/ml ETA or 2.5 μg/ml ADA, as well as their isotype control antibodies (human IgG_1; F) before incubation with IFN-λ3. After 18 (D and E) and 42 h (E), CXCL10 mRNA expression (D) and production (E) were evaluated and results displayed as absolute values without or with TNFi. Isotype control antibodies for TNFi did not affect the production of CXCL10/IP-10 induced by IFN-λ3, as illustrated by F, displaying 1 of the experiments reported in E. Significant increases: $^*P < 0.05$, $^{**}P < 0.01$.

proapoptotic effect exerted by DEX in pDCs [17] to enhance their CCR7, CD62L, CD80, CD83, ICOS-L, and MHC-I expression levels [16, 17]; to reduce IL-10, IL-13, and IFN-γ production by cocultures of pDCs with allogenic T cells [16]; and to potentiate the production of IFN-α induced by CpG-A [18]. Interestingly, a comparison of the potency between IFN-λ3 and IFN-λ1, used at identical concentrations, revealed that they were essentially equivalent in terms of promotion of survival, antigen modulation, and cytokine production in pDCs and that they do not function additively/synergistically.

Notably, analysis of the patterns of IFN-α and CXCL10/IP-10 production by pDCs incubated with IFN-λ3 uncovered a number of peculiar features. For instance, in some but not all donors, we noticed that CXCL10/IP-10 was produced by IFN-λ3-treated pDCs before IFN-α, therefore indicating that CXCL10/IP-10 might not be necessarily dependent on IFN-α, as also reported to occur in pDCs incubated with CpG [52]. In fact, whereas maximal IFN-α yields were always detected after 42 h of pDC incubation with IFN-λ3, peak levels of CXCL10/IP-10 were observed to occur, depending on the donor, earlier. Another observation that intrigued us was the large variability in the amounts of IFN-α and CXCL10/IP-10 detectable in supernatants harvested from IFN-λ3-treated pDCs (ranging from a few to thousands of picograms/milliliter), which we initially considered "expectable," given the use of primary cells. However, a more diligent, retrospective scrutiny of our data globally revealed that healthy donors could be differentiated into 3 groups based on the kinetics and the amounts of CXCL10/IP-10 produced by their IFN-λ3-treated pDCs: 1) 1 group, including donors whom we defined low CXCL10/IP-10 producers, whose IFN-λ3-treated pDCs release modest quantities of CXCL10/IP-10; 2) a second group, including donors whom we called 18 h strong CXCL10/IP-10 producers, whose IFN-λ3-treated pDCs produce remarkably elevated levels of CXCL10/IP-10 already after 18 h; 3) and a third group, including donors whom we called 42 h strong CXCL10/IP-10 producers, characterized by pDCs expressing very high CXCL10/IP-10 levels at the 42 h time point. Such patterns recall observations made in a previous study describing that PBMCs from healthy donors appeared to function as "early" or "late" responders to IFN-λ1: early responders showed peak mRNA levels for CXCL9, CXCL10, and CXCL11 between 15 and 240 min, whereas late responders peaked between 24 and 72 h [53]. Concomitantly, 2 types of "IFN-α producers" could also be distinguishable in our study, namely those whose IFN-λ3-treated pDCs produce IFN-α at levels <150 pg/ml and those producing the cytokine at levels >500 pg/ml. Interestingly, whereas all 42 h strong CXCL10/IP-10 producers corresponded to the donors whose pDCs produced high levels of IFN-α (>500 pg/ml), the 18 h strong CXCL10/IP-10 producers did not. Altogether, data depict a very complex scenario, implying that donor-dependent factors might likely condition, in a differential manner, the production of IFN-α and CXCL10/IP-10 by IFN-λ3-treated pDCs. The molecular bases underlying the variable capacity of pDCs to produce IFN-α and CXCL10/IP-10 by the various donor typologies and its potential biologic implication(s) are unknown and need to be investigated better. In such regard, a number of polymorphisms are present at the level of the IFN-α [54] and CXCL10 [55–57]

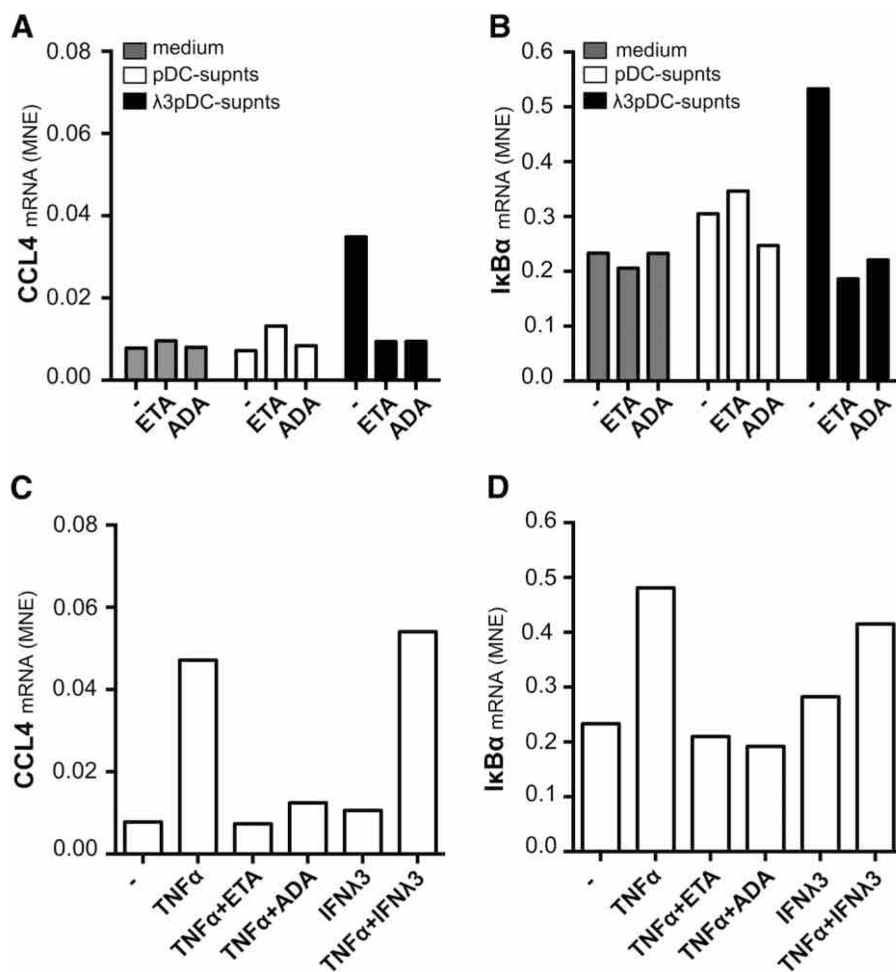

Figure 8. Supernatants harvested from IFN-λ3-stimulated pDCs exert TNF-α-dependent biologic activities in CD14⁺ monocytes. pDCs were cultured for 18 h with or without 30 ng/ml IFN-λ3. Then, cell-derived supernatants were harvested (pDC-supnts for unstimulated pDCs, λ3pDC-supnts for IFN-λ3-treated pDCs), diluted 1/1 with tissue-culture medium, and then transferred to CD14⁺ monocytes in the presence or absence of ETA or ADA (A and B). Concomitantly, CD14⁺ monocytes were also incubated in the presence or absence of 30 ng/ml IFN-λ3 or 0.1 ng/ml TNF-α, the latter cytokine alone or in presence of ETA, ADA, or IFN-λ3 (C and D). After 1 h of incubation, CCL4 (A and C) and IκBα (B and D) mRNA expression was evaluated by RT-qPCR. Each panel reports a representative experiment out of 3 with similar results.

loci, in turn, influencing the expression/production of the related products in different diseases, including sarcoidosis [54], HBV [55], tuberculosis [56], and malaria [57]. However, preliminary results would exclude the existence of a correlation among the 3 groups of CXCL10/IP-10 producers by us identified and 2 of the polymorphisms identified in the CXCL10 promoter region, namely the −1447A > G and −135G > A ones [55, 57] [unpublished results]. Moreover, it would also be worthy to investigate whether and how the 3 CXCL10/IP-10 producer groups associate with those SNPs detectable near IFN-λ3, which in HCV-infected patients, are predictive for a failure in responding to the peg-IFN-α/ribavirin therapy or in spontaneously clearing HCV infection [6–9, 58].

Whatever the case is, experiments that used antibodies neutralizing the IFN-αR definitively confirmed that the production of CXCL10/IP-10 triggered by IFN-λ3-treated pDCs, isolated from the 42 h strong CXCL10/IP-10 producers, is scarcely dependent on endogenous IFN-α. Even though no 18 h strong CXCL10/IP-10 producers could be included in IFN-αR-neutralizing experiments, based on the observations described above, there is no reason to believe that production of CXCL10/IP-10 by their IFN-λ3-treated pDCs may be more dependent on endogenous IFN-α than in 42 h strong CXCL10/IP-10 producers. By contrast, the use of different TNF-α inhibitors, namely, ADA, ETA, and infliximab [49], allowed us to uncover that the expression of CXCL10/IP-10 by IFN-λ3-treated pDCs is totally driven by endogenously produced TNF-α, regardless of the CXCL10/IP-10 producer group. Under the same experimental conditions, endogenous TNF-α was found to contribute slightly to the production of IFN-α [unpublished results] and not to affect pDC survival, pointing to its distinctive role in promoting CXCL10/IP-10 expression. Moreover, no inhibition of CXCL10/IP-10 expression by ETA was observed in pDCs incubated with R837 (which produces massive amounts of TNF-α, IFN-α, and CXCL10/IP-10) [23, 46, 59], thus excluding a "reverse signaling"-mediated inhibitory effect [60] and indicating an apparently specific effect of endogenous TNF-α under IFN-λ3 treatment only. Intriguingly, notwithstanding the results obtained by TNF-α blockers, exogenous TNF-α alone, at doses ranging from 0.01 to 10 ng/ml, triggered only minute amounts of CXCL10/IP-10 in pDCs, even though it induced, for instance, CXCL8 mRNA and pDC maturation [unpublished results]. On the other hand, supernatants harvested from pDCs treated with IFN-λ3 for 18 h displayed the capacity to induce, in a TNF-α-dependent manner, the expression of CCL4 and IκBα mRNAs in CD14⁺ monocytes, thus proving definitively that the TNF-α, present in pDC-derived supernatants, is biologically active.

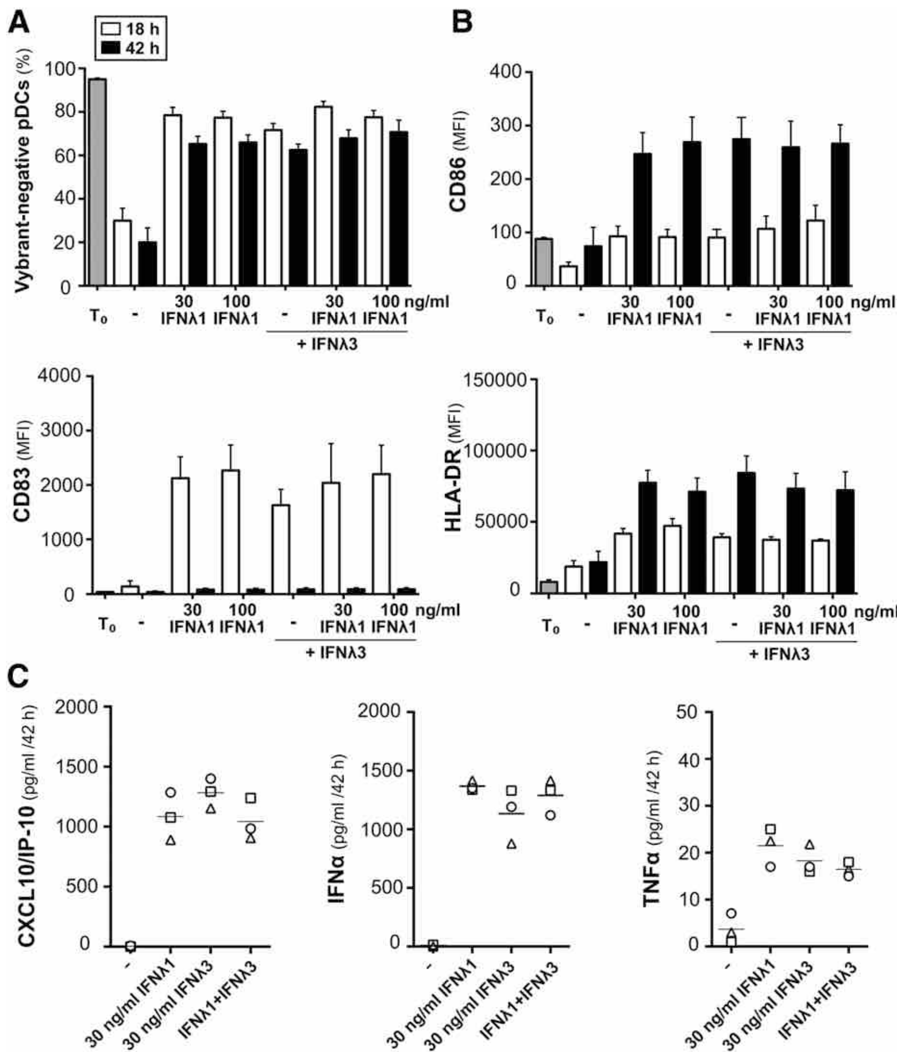

Figure 9. IFN-λ1 and IFN-λ3 are equally efficient in promoting survival, antigen modulation, and cytokine production in pDCs. Freshly isolated pDCs were incubated in the presence or absence of 30 ng/ml IFN-λ3 with or without 30 or 100 ng/ml IFN-λ1. After 18 and 42 h, pDCs were harvested and analyzed for viability (A) or HLA-DR, CD86, and CD83 expression (by use of Vybrant DyeCycle; B) by flow cytometry. For each antigen, bar graphs report the results as MFI, calculated after subtracting the MFI given by the correspondent isotype control antibodies or the basal fluorescence (*n* = 3). (C) Absolute production of CXCL10/IP-10, IFN-α, and TNF-α, measured in supernatants from pDCs incubated for 42 h. Data are from the same 3 donors reported in A and B.

A number of reasons might explain why endogenously produced TNF-α is able to control the production of CXCL10/IP-10 in pDCs incubated with IFN-λ3. One possibility might be that the CXCL10 locus, following treatment with IFN-λ3, but not spontaneously, undergoes a chromatin reorganization that becomes accessible to the signals triggered by endogenous TNF-α. If so, such a scenario would resemble what occurs in human neutrophils with regard to their production of IL-6 in response to R848 and/or TNF-α [61]. An alternative, more likely explanation, relies instead on the known molecular mechanisms controlling an optimal CXCL10 transcription, which is well demonstrated to involve a synergistic action of the transcription factors STAT and NF-κB [43–45]. According to such an explanation, we would speculate that STATs and NF-κB would be mobilized to the CXCL10 promoter in pDCs incubated with IFN-λ3: STATs directly in response to IFN-λ3 and NF-κB following activation by endogenously produced TNF-α.

In conclusion, our study greatly extends our knowledge on the biologic effects that IFN-λ3 exerts on pDCs, which might be relevant in the context of viral infections, particularly in the case of HCV progression. Accordingly, if pDCs are recruited into the liver of chronic hepatitis C patients [62], then it is tempting to speculate that locally produced IFN-λ3 activates pDCs to express ISG genes and to produce TNF-α, IFN-α, and CXCL10/IP-10. Locally, TNF-α may contribute to hepatic inflammation and cell death, whereas CXCL10/IP-10 and IFN-α could influence the recruitment and activation of $CXCR3^+$ cells (such as monocytes, NK cells, and Th1 cells) [27] in the infected liver. Conceivably, these phenomena would all sustain the progression of inflammation and immune response, eventually leading to chronic infection. More broadly, our data also suggest that IFN-λ3, by triggering the production of IFN-α, CXCL10/IP-10, and TNF-α by pDCs, may impact on the cytokine balance controlling the polarization/recruitment of Th cells, favoring, in turn, the Th1 phenotype, in line with what was previously shown for IFN-λ1 and IFN-λ2 [63, 64].

AUTHORSHIP

G. Finotti, N.T., G. Fattovich, and M.A.C. conceived of and designed the experiments. G. Finotti, F.C., and N.T. performed the experiments and analyzed the data. M.A.C. wrote the paper.

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DISCLOSURES

The authors declare no conflicts of interest.

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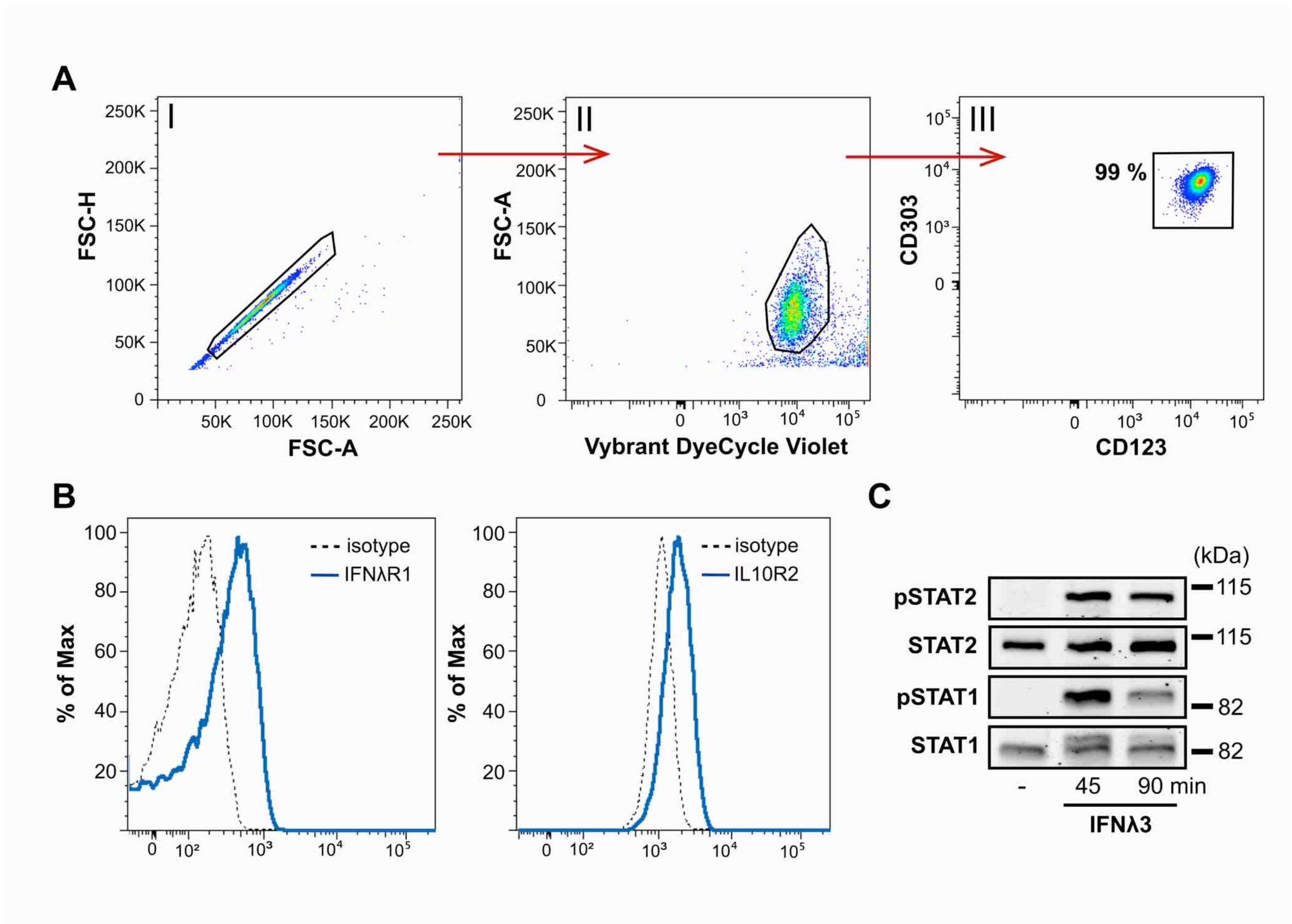
KEY WORDS:
pDCs · innate immunity · interferons · antiviral response

Results (i)

2.2.2 SUPPLEMENTAL MATERIAL FOR:

Endogenously produced TNFα contributes to the expression of CXCL10/IP-10 in IFN-λ3-activated plasmacytoid dendritic cells

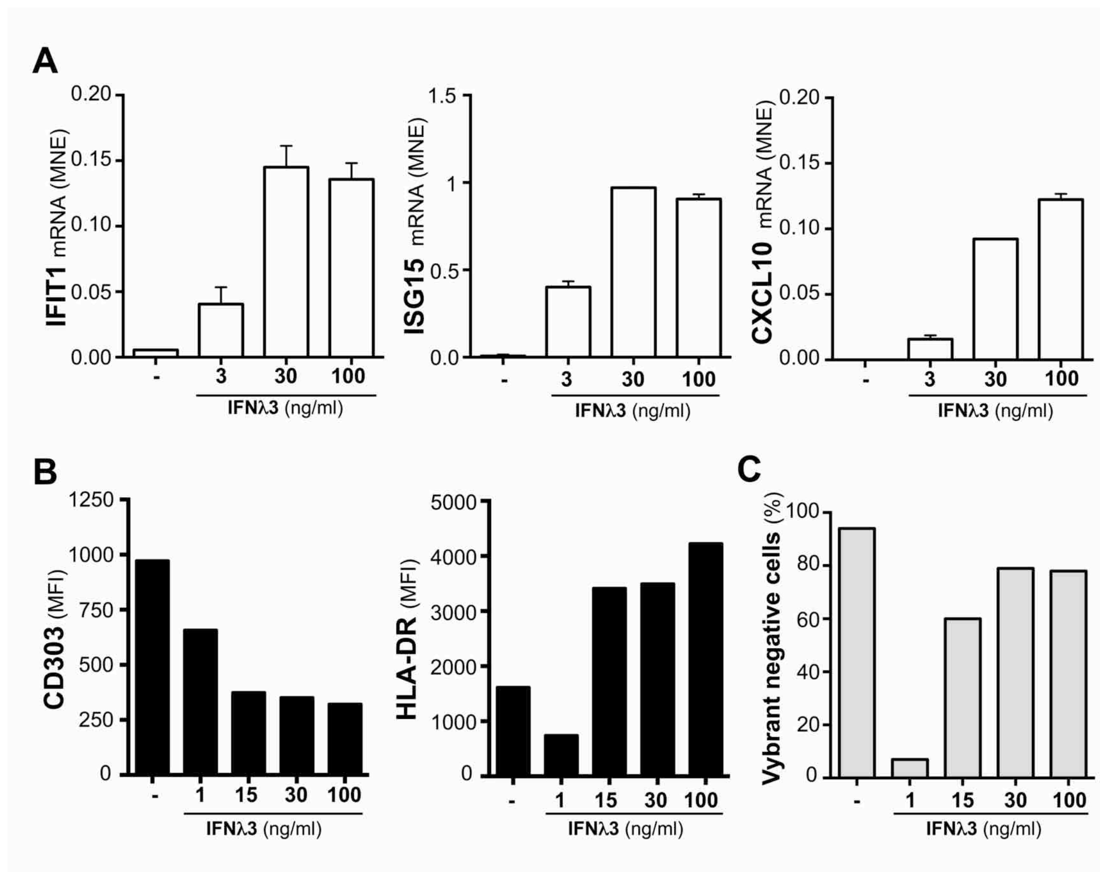
Results (i)



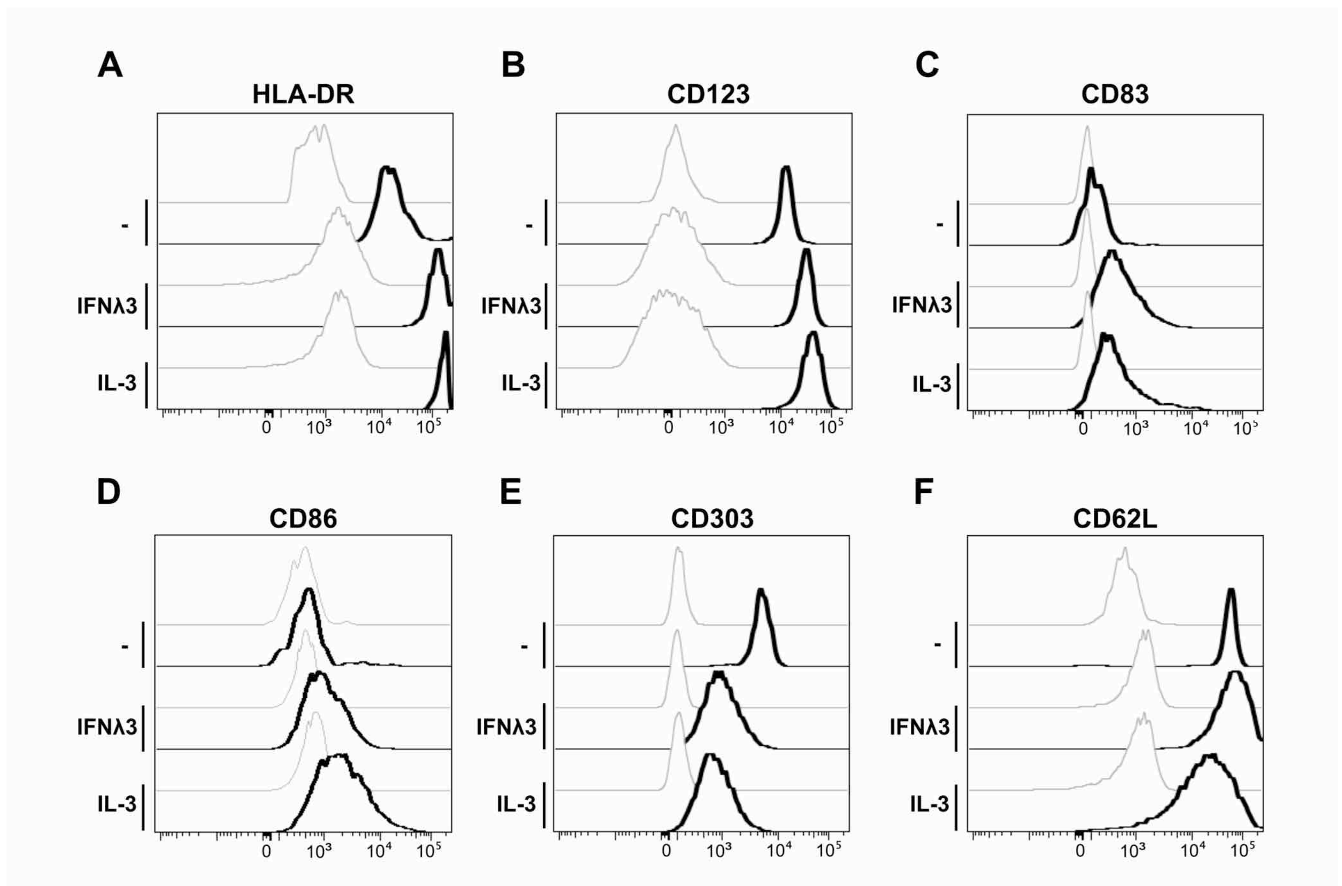
Supplemental Figure 1. Purity, IFNλR expression and IFNλ3 responsiveness by human plasmacytoid cells (pDCs).

pDCs were isolated from peripheral blood of healthy volunteers by immunomagnetic separation (see M&M) and then stained with antibodies towards CD303 and CD123 (**A**). Panels show representative plots of the overall gating strategy for FACS analysis. Live cells were selected by firstly gating on singlet cells (panel **I**), and then on Vybrant™ DyeCycle™ Violet-negative cells (panel **II**). Marker modulation and/or viability assays were performed on this cell population (panel **II**). pDC purity was determined gating on $CD303^{+}/CD123^{+}$-live cells (panel **III,** always > 98 %).

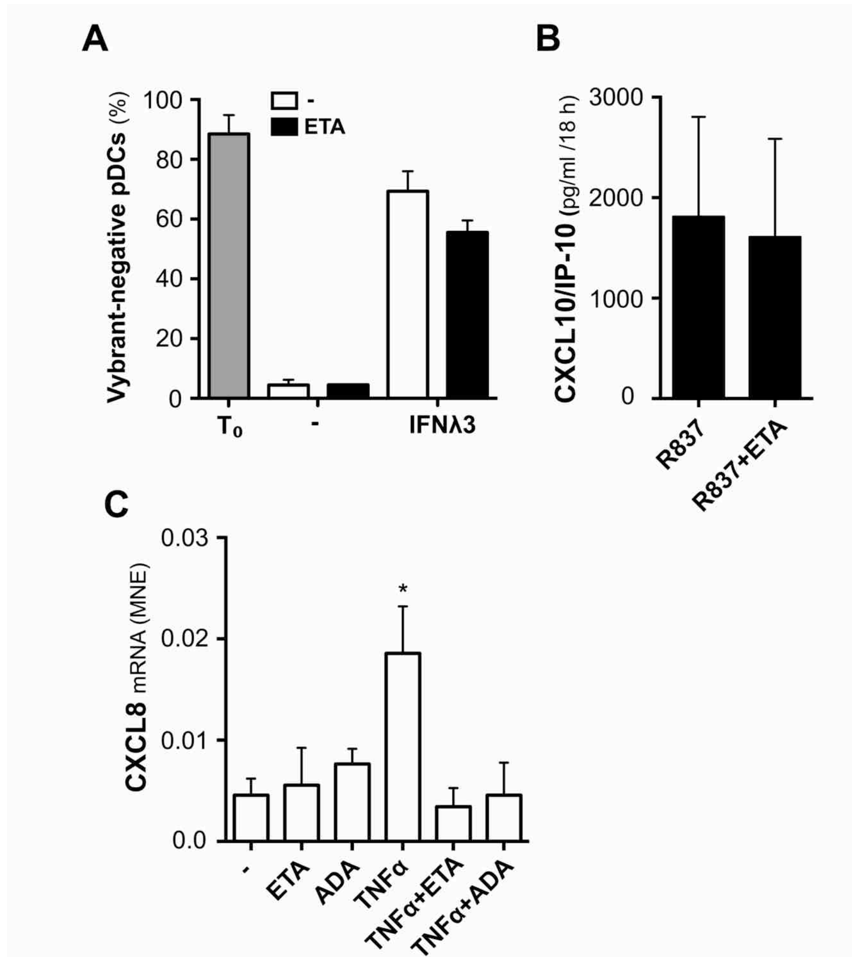
Panels in (**B**) display flow cytometric plots illustrating IFNλR1 and IL-10R2 expression levels in freshly isolated pDCs. Panel (**C**) displays an immunoblot demonstrating that a strong STAT1 and STAT1 tyrosine phosphorylation is induced in pDCs incubated with 30 ng/ml IFNλ3 for 45 and 90 min. Panels (**B**) and (**C**) show one representative experiment out of, respectively, five and three performed with similar results.



Supplemental Figure 2. pDCs are activated by IFNλ3 in a dose-dependent manner.
Freshly isolated pDCs were incubated with different concentrations of IFNλ3 for 18 h and then evaluated for IFIT1, ISG15 and CXCL10 mRNA induction by RT-qPCR (panels in **A**), HLA-DR and CD303 expression by flow cytometry (panels in **B**), and survival by Vybrant^{TM}DyeCycle^{TM}Violet assay (panel **C**). (**A**) Gene expression data (mean ± SEM) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. (**B**) For each antigen, expression is displayed as net mean fluorescence intensity (MFI) after subtraction the correspondent basal fluorescence. (**C**) Viability was assessed by flow cytometry analysis, gating on Vybrant^{TM}DyeCycle^{TM}Violet -negative cells to exclude apoptotic cells (**A**). (**A-C**) Representative experiments out of at least three performed with similar results.



Supplemental Figure 3. Expression of surface markers in pDCs incubated with IFNλ3 or IL-3. Freshly isolated pDCs were incubated with or without 30 ng/ml IFNλ3 or 20 ng/ml IL-3 for 42 h and then analysed for HLA-DR (**A**), CD123 (**B**), CD83 (**C**), CD86 (**D**), CD303 (**E**) and CD62L (**F**) expression by flow cytometry. Panels show histograms for each marker as compared to the corresponding basal fluorescence. Black lines stand for marker fluorescence, while gray lines stand for basal fluorescence. One representative experiment.



Supplemental Figure 4. Role of endogenous TNFα in mediating cell viability or CXCL10 production in, respectively, IFNλ3- or R837-treated pDCs.

(**A**) pDCs were pretreated for 30 min with or without 5 µg/ml etanercept (ETA) prior to incubation with IFNλ3. After 42 h, cells were harvested and stained by Vybrant™DyeCycle™Violet to assess their viability by flow cytometry analysis (see M&M). Bars in the graphs show the means ± SEM (n=5) of alive pDCs. (**B**) pDCs were pretreated with or without 5 µg/ml ETA and then incubated with 5 µM R837. After 18 h, cells were harvested, centrifuged and their supernatants evaluated for CXCL10 content by ELISA (n=3). (**C**) pDCs were pretreated for 30 min with or without 5 µg/ml etanercept or or 2.5 µg/ml adalimumab, and then incubated with or without or 10 ng/ml TNFα. After 3 h, cells were lysed for total RNA extraction and CXCL8 mRNA expression evaluated by RT-qPCR. Gene expression data are depicted as MNE units after RPL32 mRNA normalization (n=3). Asterisks in (**C**) indicate significant variations of TNFα-stimulated sample compared to the other stimulatory conditions: *$P<0.05$.

III. SECOND AIM OF THE STUDY

Second aim of the study

TASK 2: pDC ACTIVATION BY IL-3: MORE THAN JUST A GROWTH FACTOR

IL-3 is a cytokine functioning as a growth factor for pDCs that belongs to the β common (βc) family of cytokines, together with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (165). The βc family of cytokines was originally identified for its ability to stimulate hematopoiesis (166). IL-3 signals through a heterodimeric cell surface receptor that is composed of a major binding, cytokine-specific subunit (IL3Rα) and a common β chain subunit. Among the many biological activities exerted by IL-3, "cell survival" is one of its key functions, specifically in maintaining hematopoietic cell viability. In fact, the βc family of cytokines has the ability to regulates the production and function of a wide spectrum of hematopoietic cells (166).

Despite its broad range of biological functions, IL-3 is not normally detected in the circulation and is largely not essential for steady-state immune functions, but can be secreted during emergency myelopoiesis, following, for example, infections (165). Under inflammatory conditions, IL-3 is secreted mainly by activated T cells, endothelial cells and mast cells (see also **Figure 5**) (165,167,168). IL-3 stimulates the production and activation of mast cells and basophils, important cellular regulators of the Th2-mediated inflammatory responses against parasites or during allergic diseases (165).

A role for IL-3 in solid tumor development has been reported (169). Indeed, not only hematopoietic cells, but also endothelial cells express receptors for IL-3 and respond to IL-3 in multiple ways (167). Recent evidence has shown that IL-3 regulates the development and progression of solid tumors through its ability to stimulate the formation of new blood vessels from endothelial cells (170).

In this context, given their high expression of CD123 (the IL-3Rα subunit)(4), pDCs are a well-known target of IL-3. pDCs, in fact, differentiate into mature DCs when incubated with IL-3 (50), as observed by an increased expression of co-stimulatory molecules CD80, CD86, and MHC-I and -II (4,49). Moreover, IL-3-stimulated pDCs produce TNFα (49,171), that in turn mediates the

maturation-promoting effect by IL-3. Finally, pDCs cultured with IL-3 acquire Ag presentation capacity, and preferentially prime Th2 cells (49). Interestingly, because of its potent prosurvival action on pDCs *in vitro* (49), IL-3 is commonly added to the culture medium during experiments in which the effects on pDCs by a given stimulus of interest is tested (41,42,172,173). Interestingly, during my studies related to my first task, I observed that IFNλ3 upregulates the expression of the IL-3Rα subunit (CD123), suggesting that, by this mechanism, it might modulate pDC responsiveness to IL-3.

Based on these premises, in this second part of my project I investigated the effect of IL-3 on pDCs, alone or in the presence of IFNλ3. To do so, I examined whether IFNλ3 and IL-3 could cooperate in activating human pDCs.

3.1 RESULTS (ii)

3.1.1 Expression of surface CD123/IL-3R and IFNλR1 are upregulated by both IFNλ3 and IL-3 in human pDCs

Preliminary flow cytometry experiments revealed that both IFNλ3 and IL-3, in addition to increase the levels of surface CD123 (**Figure 13A**) (174), also upregulate the expression of IFNλR1 in human pDCs incubated for 18 h (**Figure 13B**), therefore indicating that they may reciprocally influence responsiveness of pDCs to each other. Since I have previously shown that either IFNλ3 or IL-3 prolong the viability of pDCs (174), I then investigated whether they could promote a more potent prosurvival effect when used in combination. However, that was not the case, because, as shown in **Figure 13C**, each cytokine by itself substantially triggered a maximal increase of pDC survival. Similarly, neither CD123 (**Figure 13A**), nor IFNλR1 (**Figure 13B**) expression was additively or synergistically enhanced by IFNλ3 and IL-3 used in combination.

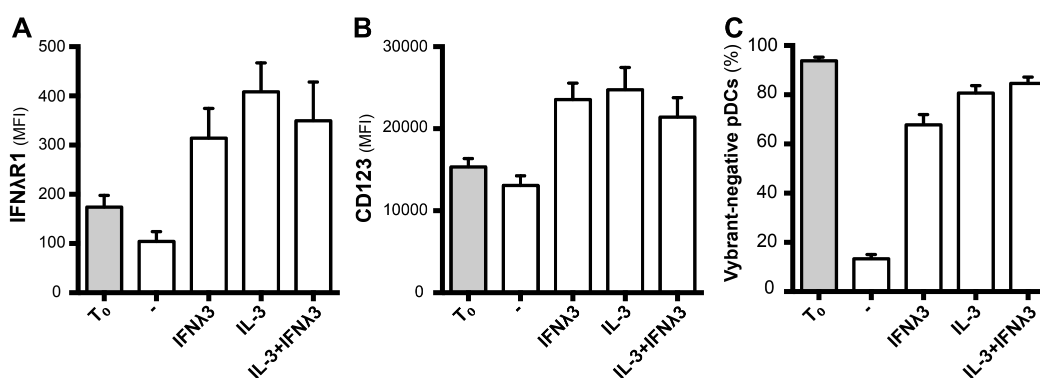


Figure 13. Effect of IFNλ3 and/or IL-3 on the IFNλ3R1 and IL-3Rα expression as well as survival by pDCs

pDCs were incubated with or without 30 ng/ml IFNλ3 and/or 20 ng/ml of IL-3. Cells were harvested after 18 h and analyzed by flow cytometry for CD123 (panel **A**) and IFN**λ**R1 (panel **B**) surface expression in comparison with freshly isolated pDCs (T_0). The bar graphs report the means ± SEM (n= 7-15). MFI was calculated after subtracting the MFI given by the correspondent isotype control antibodies or the basal fluorescence. In the same experiments, pDCs were stained with the Vibrant DyeCycle to assess their viability (panel **C**). Gating strategy to identify live pDCs has been performed as previously described (174).

3.1.2 IFNλ3 and IL-3 synergistically induce the production of IFNα by human pDCs

Subsequently, I analyzed the production of IFNα by measuring its accumulation in cell-free supernatants from pDC cultures incubated with or without IFNλ3 and/or IL-3 for up to 18 h. As shown in **Figure 14** (panel **A** displaying the values of all individual donors; panel **B** the related means ± SEM), minimal amounts of IFNα (less than 10 pg/ml) were detected from pDCs treated with either IFNλ3 or IL-3, but not in their absence. By contrast, a synergistic production of IFNα was instead measured after 18, but not 5, h of pDC incubation with both IFNλ3 and IL-3 (**Figure 14A** and **B**), which was also observed at gene expression level by qPCR (**Figure 14C**). In these latter experiments, IFNλ3 and IL-3 alone were found to modestly, but significantly induce IFNα mRNA accumulation at the 18 h time point only (**Figure 14C**). Under the same experimental conditions, IL-3 was also found to upregulate the mRNA expression of various interferon responsive genes (ISGs), such as IFIT1 (Interferon-Induced Protein with Tetratricopeptide Repeats 1), ISG15 (ISG15 Ubiquitin-Like Modifier) and MX1 **(**Myxovirus Resistance 1**)** after 18 h of pDC incubation, while IFNλ3 was found to trigger a similar effect already after 5 h (**Figure 14D**). Notably, also ISG mRNA expression was synergistically upregulated by cotreatment of pDCs with IFNλ3 and IL-3 at the 5 and 18 h time-points, with the exception of IFIT1 mRNA, which was synergistically augmented at the 5 h time-point (**Figure 14D**).

To clarify if, and to what extent, endogenous IFNα could be responsible for the induction of ISG mRNA expression in pDCs treated with IL-3 and/or IFNλ3, I performed new experiments using antibodies (Abs) neutralizing the IFNαR. As shown in **Figure 14E**, upregulation of IFIT1, ISG15 and MX1 mRNA expression in pDCs treated with IL-3 for 18 h was largely blocked by the αIFNαR Abs, suggesting a functional autocrine action by endogenous IFNα. By contrast, ISG expression induced in pDCs either by IFNλ3 alone, or by IFNλ3 plus IL-3, was only slightly affected by the αIFNαR Abs (**Figure 14E**), indicating a major role of IFNλ3 in directly triggering the expression of IFIT1, ISG15 and MX1.

Taken together, data demonstrate that, in pDCs, IL-3 induces the production of small, but biologically active amounts of IFNα, which autocrinally induces the expression of ISG mRNAs. Data also demonstrate that IFNλ3 plus IL-3 synergize in inducing the production of IFNα, as well as the expression of ISG mRNA: the latter phenomenon, however, occurs independently from endogenous IFNα.

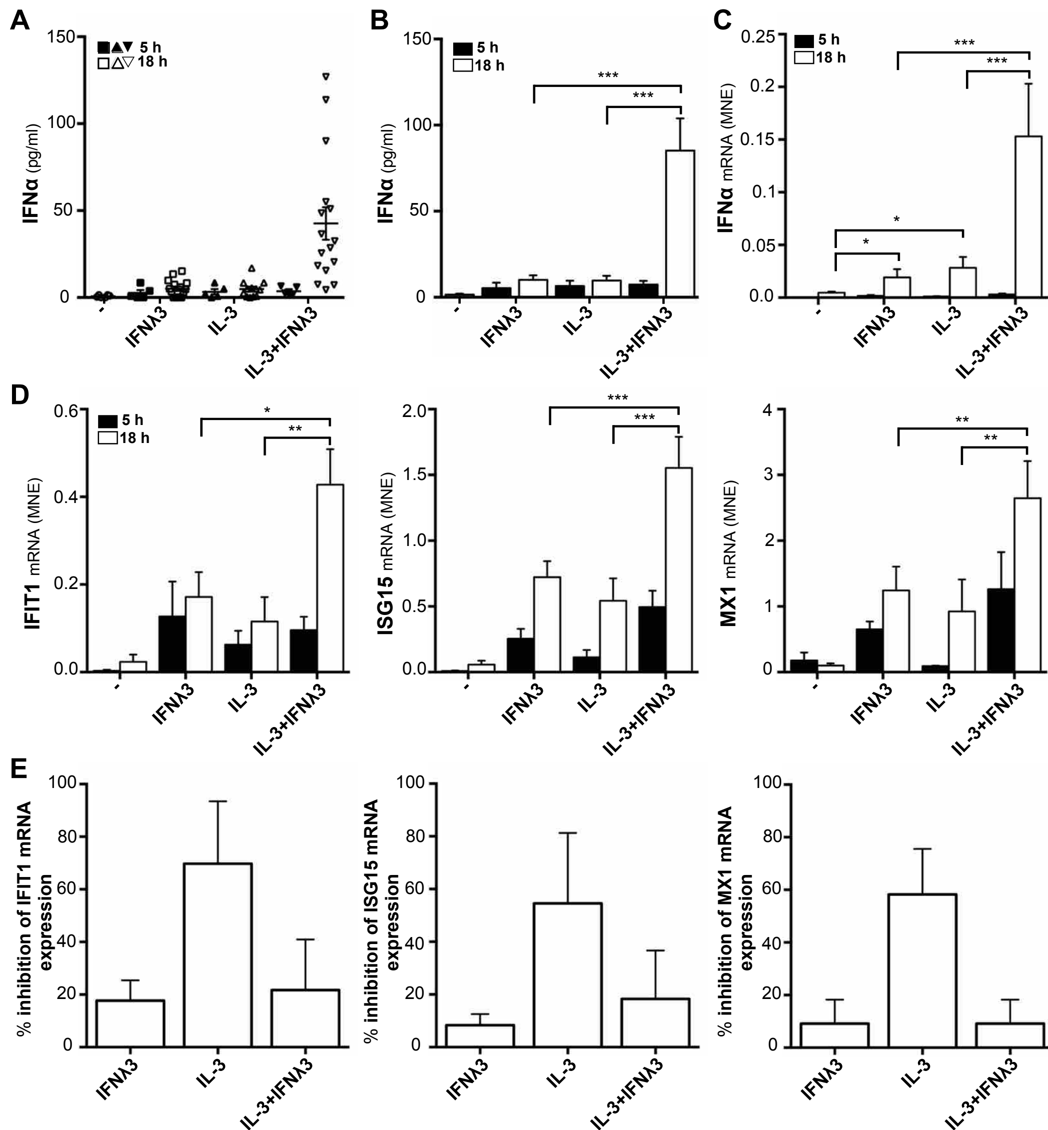


Figure 14. **Production of IFNα by pDCs treated with IL-3 plus IFNλ3 and its involvement in mediating ISG mRNA expression**

After isolation, pDCs were incubated with or without 30 ng/ml IFNλ3, 20 ng/ml IL-3 or their combination for 5 and 18 h. Cell-free supernatants were collected and extracellular IFNα (panels **A** and **B**) measured by ELISA. Panel (**A**) shows the results of all individual experiments for the displayed conditions, while panel (**B**) their related means ± SEM (n= 5-16). No IFNα was measurable in supernatants from pDCs cultured up to 18 h in the absence of stimuli. (panels

C and **D**), pDCs were incubated for 5 and 18 h and evaluated for IFNα (**C**), IFIT1, ISG15 and MX1 (**D**) mRNA expression by RT-qPCR. Results (mean ± SEM, n=5-14) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. (panel **E**) pDCs were incubated with or without 5 μg/ml of αIFNαR or mouse IgG_{2a} (isotype control antibodies, not shown) for 30 min and then incubated with IFNλ3, IL-3 or their combination for 18 h. Bar graphs show the percentage of inhibition of IFIT1, ISG15 and MX1 mRNA expression (means ± SEM, n= 4) exerted by αIFNαR antibodies. Under the same experimental conditions, isotype control antibodies did not affect mRNA expression of these genes (data not shown). Significant variations: * P<0,05; ** P <0,01; *** P <0,001.

3.1.3 IFNλ3 and IL-3 synergistically induce the production of TNFα by human pDCs independently from IFNα

Measurement of TNFα in the same pDC-derived supernatants confirmed (174) that IFNλ3 induces the production of very low, but detectable, levels of the cytokine (**Figure 15A** and **B**). Similarly, IL-3 confirmed (171) to trigger a remarkable production of TNFα by pDCs (**Figure 15A** and **B**), already after 5 h of incubation in line with the results from gene expression experiments (**Figure 15C**). Interestingly, the amounts of TNFα detected in supernatants from IFNλ3 plus IL-3-treated pDCs were significantly higher than those from IL-3-treated pDCs, but only at the 18 h time-point (**Figure 15A** and **B**), consistent with findings at the TNFα mRNA level (**Figure 15C**). Expression of TNFα mRNA in pDCs treated with IL-3 or IFNλ3 alone was instead maximal after 5 and 18 h, respectively (**Figure 15C**). Given that endogenous IFNα was shown to be involved in supporting TNFα production in IL-3 plus CpG-stimulated pDCs (41), I investigated its role in our experimental system by incubating pDCs in the presence of αIFNαR Abs for 18 h. As shown in **Figure 15D**, the production of TNFα by IFNλ3 and/or IL-3-stimulated pDCs was minimally influenced by the presence of αIFNαR Abs, suggesting that endogenously produced IFNα is substantially dispensable for the generation of TNFα, independently from its yields.

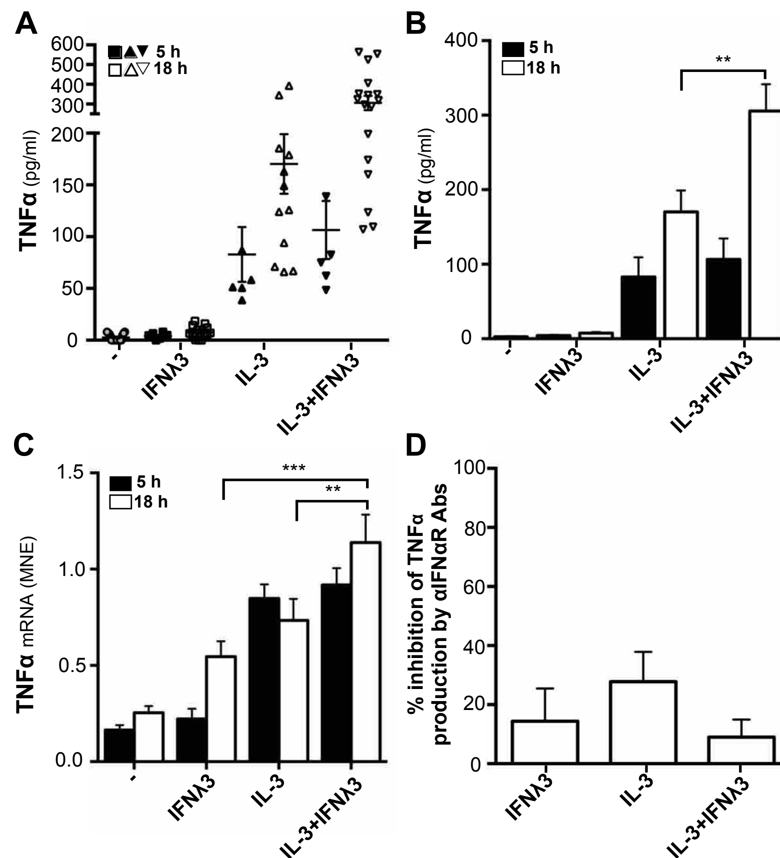


Figure 15. Synergistic production of TNFα by pDCs incubated with IL-3 plus IFNλ3 pDCs were incubated with or without 30 ng/ml IFNλ3, 20 ng/ml IL-3, or their combination, for 5 and 18 h to analyze TNFα protein production (panels **A** and **B**) or gene expression (panel **C**). (**A**) Results from all individual measurements by ELISA. (**B**) means ± SEM of A (n= 5-17). (**C**) Expression of TNFα mRNA in pDCs. Gene expression data (mean ± SEM, n=8-14) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. (panel **D**) pDCs were pretreated for 30 min with or without 5 μg/ml αIFNαR or mouse IgG_{2a} (isotype control antibodies, not shown) and then incubated with IFNλ3, IL-3, or their combination for 18 h. Bar graphs shows the percentage of inhibition on TNFα production (n= 3-5) exerted by αIFNαR antibodies. Under the same experimental conditions, isotype control antibodies did not affect TNFα production (data not shown). Significant variations: ***P*<0,01; ****P*<0,001.

3.1.4 Endogenous TNFα is required for IFNα production by IFNλ3- and/or IL-3-treated pDCs

Because it has been previously shown that, in primary or synovial macrophages from patients with rheumatoid arthritis, TNFα may function as an endogenous inducer of type I IFN production (104,175), I investigated whether the same could occur under our experimental conditions. As shown in **Figure 16**, etanercept

(ETA), a TNFα blocker (176), potently inhibited both IFNα mRNA expression (**Figure 16A**) and IFNα production (**Figure 16B**) in pDCs treated with IFNλ3 plus IL-3 for 18 h. Similar results were observed when pDCs were incubated with IFNλ3 plus IL-3 in the presence of adalimumab (ADA), another TNFα blocker (data not shown). It was not possible to precisely quantify the effect of ETA on IFNλ3- or IL-3-stimulated pDCs, due to the scarce amounts of IFNα produced by them. However, expression of IFNα mRNA was almost completely abrogated by ETA in pDCs treated with either IFNλ3 or IL-3 (data not shown). Nonetheless, ETA almost completely blocked IFIT1, ISG15 and MX1 mRNA expression in pDCs treated with IFNλ3 plus IL-3 (**Figure 16C**), consistent with the results shown in **Figure 16A**. ETA also diminished the expression of IFIT1 and ISG15 mRNA induced by IFNλ3 alone (**Figure 16C**), exerting a higher suppressive effect in IL-3-treated cells (**Figure 16C**). Importantly, specificity of ETA effects on IFNα and ISG expression was demonstrated by its inability to significantly influence the prosurvival action by IFNλ3 and/or IL-3 in pDCs (**Figure 16D**). In final experiments, TNFα exogenously added to pDC cultures was found to directly induce either the production of IFNα (**Figure 16E**), at levels similar to IFNλ3 or IL-3 (see **Figure 14A**), or a rapid expression of ISG15 mRNA (**Figure 16E**).

Altogether, data demonstrate that the synergistic production of IFNα by pDCs incubated with IFNλ3 plus IL-3 is mainly mediated by endogenous TNFα, which is synergistically induced by the two stimuli in combination, and that by itself results able to directly trigger IFNα expression.

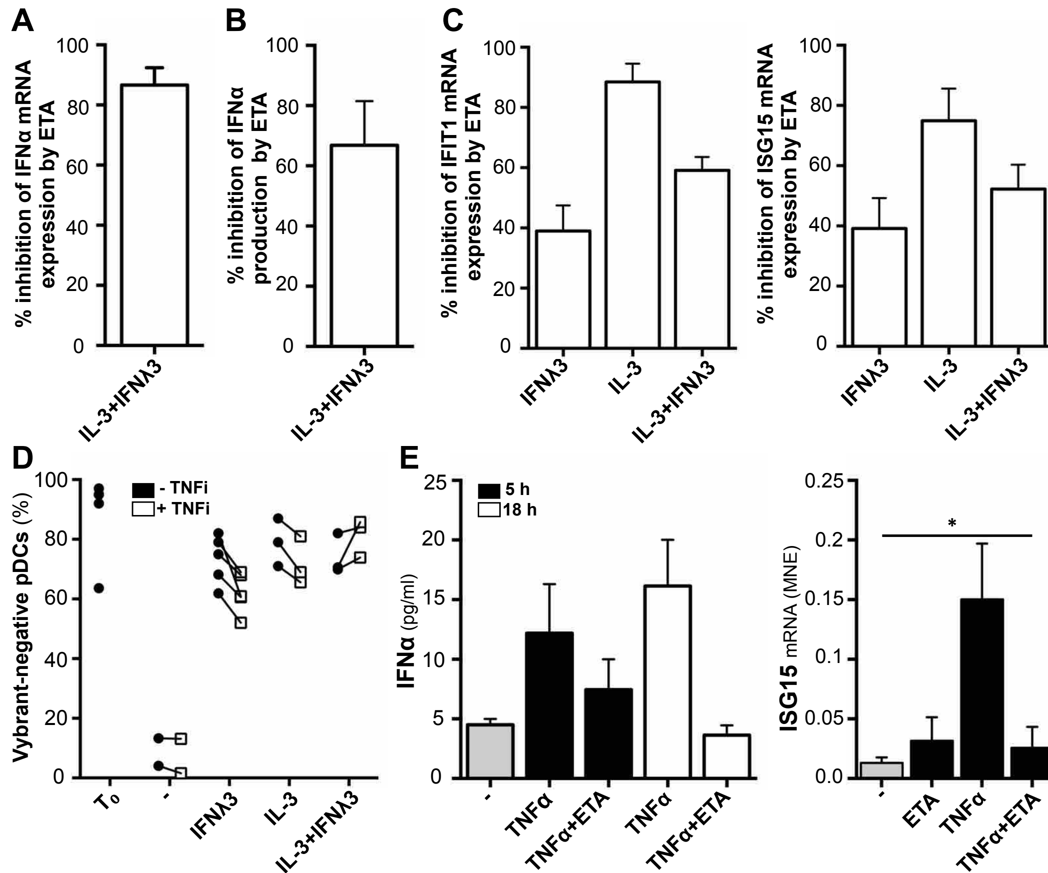


Figure 16. Role of endogenous TNFα in mediating the production of IFNα and the mRNA expression of ISGs in pDCs treated with IL-3 plus IFNλ3

pDCs were pretreated with or without 5 μg/ml ETA or human IgG_1 (isotype control antibodies) for 30 min, and then incubated with IL-3 plus IFNλ3 for 18 h. Bar graphs shows the percentage of inhibition of IFNα mRNA expression (panel **A**) and IFNα production (panel **B**) (means ± SEM, n= 3-4) exerted by ETA. Under the same experimental conditions, isotype control antibodies did not affect IFNα production or mRNA expression (data not shown). (panel **C**) pDCs were pretreated with or without 5 μg/ml ETA and then incubated with IFNλ3, IL-3 or their combination for 18 h. The percentage of inhibition on IFIT1 and ISG15 mRNA expression is reported (means ± SEM, n= 3). (panel **D**) pDCs were pretreated with ETA prior to stimulation, and after 18 h harvested and stained by Vybrant DyeCycle Violet to assess their viability by flow cytometry. Bars in the graphs show the means ± SEM (n=4) of live pDCs in the presence or absence of ETA. Gating strategy to identify live pDCs has been performed as previously reported (174). (panel **E**) pDCs were cultured in the presence or absence of 10 ng/ml TNFα, for 5 and 18 h, to measure their capacity to produce IFNα (by ELISA, mean ± SEM, n= 4). (panel **F**) IFIT1 mRNA expression in pDCs treated with 10 ng/ml TNFα for 3 h. Gene expression data (mean ± SEM, n=4) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. Significant variations: **P*<0,05**.**

3.2 DISCUSSION (ii)

In this study, I investigated whether the capacity of IFNλ3 to activate various functions of human pDCs could be influenced by IL-3, since our previous experiments uncovered that IFNλ3 increases the expression of CD123, the alpha chain of the IL-3 receptor (174). Interestingly, I subsequently found that, viceversa, pDCs incubated with IL-3 augment the expression of IFNλR1. This latter observation thus strengthened my initial hypothesis that pDCs may respond more powerfully to IFNλ3 and IL-3 together, given that the two cytokines reciprocally upregulate their receptor expression. And, as described below, that was the case. In particular, I found a synergistic production of IFNα by pDCs incubated for 18 h with IFNλ3 plus IL-3, while minimal amounts of IFNα were detected from pDCs treated with either IFNλ3 or IL-3 alone. Quantitative PCR experiments demonstrated that such effects of IFNλ3 plus IL-3 were mirrored by an accumulation of IFNα mRNA, indicating actions presumably at the transcriptional level. I did not investigate, however, the intracellular signaling pathways whereby IFNλ3 plus IL-3 synergistically trigger the transcription of IFNα. It might be possible that IFNλ3 positively regulates the pathways involved in IFNα production by IL-3-treated pDCs, namely the PI3K- and MAPK-signaling cascades (177,178). Alternatively, there might be effects of the two cytokines at the post-transcriptional level, for instance on IFNα mRNA stability or translation, or even at chromatin level, favoring an increase accessibility of the transcriptional machinery at the IFNα locus. Whatever the case is, since both IFNλ3 and IL-3 alone are able to actively induce IFNα mRNA expression and production in pDCs, I would favor the notion that the different signaling pathways triggered by the two cytokines ultimately converge to synergistically increase IFNα gene transcription and protein production.

Since my experiments uncovered that the also expression of mRNAs encoding ISGs, including IFIT1, ISG15 and MX1 was synergistically increased in pDCs treated with IFNλ3 plus IL-3, I asked whether this phenomenon could be

mediated by endogenously produced IFNα. Unexpectedly, ISG mRNA expression was found to be only slightly decreased when IFNλ3 plus IL-3-treated pDCs were incubated in the presence of IFNαR neutralizing antibodies. Such a minor role of endogenous IFNα in inducing ISG mRNAs could be explained by the fact that the latter genes can be directly upregulated by IFNλ3, which indeed triggers signaling pathways similar to those activated by type I IFN (119). Accordingly, IFNαR blocking experiments did not influence the significantly upregulated expression of the various ISG mRNAs in pDCs incubated with IFNλ3 alone. On the other hand, I found that IL-3, by itself, upregulated ISG mRNA expression at levels comparable to those induced by IFNλ3 alone, and, surprisingly, in a fashion totally dependent on endogenous IFNα. Low amounts of IFNα (less than 10 pg/ml) could be, in fact, detectable in supernatants harvested from pDCs cultured with IL-3 for both 5 and 18 h. The latter data are, by the way, consistent with previous findings demonstrating that a given cytokine, even if produced in very low amounts, is potentially able to induce a remarkable cell response (104,174). Taken together, my data suggested that the synergistic increase of ISG mRNA expression in pDCs incubated with IFNλ3 plus IL-3 derives, in part, from the a presumably direct effect of IFNλ3, and, in part, from an action triggered by IL-3 mediated *via* endogenous IFNα.

It has been previously shown that pDCs incubated with IL-3 can produce variable amounts of TNFα (49,171). Moreover, pDCs incubated with RNA-containing immune complexes (IC) in the presence of GM-CSF, that shares with IL-3 a common β-receptor for signaling, were found to produce synergistic amounts of both IFNα and TNFα as compared to RNA-IC alone (168). Furthermore, in our previous work we found that also IFNλ3 induces the production of low, but biologically active, levels of TNFα by human pDCs (174). These observations prompted me to subsequently analyze whether IFNλ3 plus IL-3 could induce the production of TNFα in amounts higher than those induced by IL-3/IFNλ3 alone. That was indeed the case, as I observed a synergistic induction of TNFα mRNA

expression and production by pDCs incubated with IFNλ3 plus IL-3, detectable after 18 h and maintained up to 42 h of culture (my unpublished observations). However, I did not investigate how such a synergistic production of TNFα occurs at molecular/biochemical levels. In such regard, it is well known that the induction of TNFα mRNA expression usually depends on stimulus-induced activation of NFκB- and/or MAPK-dependent pathways (179,180), and that IL-3 is presumably able to activate NFκB (165). Similarly, and as already proved for type I IFNs (181), also IFNλ3 is able to activate NFκB- and/or MAPK-dependent signaling pathways according to some studies (147,182). Thus, I would speculate that, in pDCs treated with IFNλ3 plus IL-3, the latter cytokine is mostly responsible to activate NF-kB/MAPK, while IFNλ3 simply potentiates the IL-3-triggered signaling, in turn leading to a strong TNFα mRNA expression and protein production.

Subsequently, I explored whether, in IL-3-treated pDCs, endogenous IFNα could have some role inducing TNFα production, similarly to its action on ISG mRNA expression. As putative control, I made these experiments using also pDCs treated with IFNλ3 plus IL-3 as, under this condition, IFNα production is synergistically increased. However, IFNαR neutralizing antibodies had only a minor, or even no, effect on the production of TNFα secreted by pDCs treated either by IL-3 alone, or by IFNλ3 plus IL-3, suggesting that, independently of its yields, endogenous IFNα is not required for the production of TNFα. Conversely, experiments performed in the presence of different TNFα inhibitors, namely etanercept (ETA)(this thesis) and adalimumab (ADA) (my unpublished observations), allowed me to uncover that the production of IFNα by IFNλ3 plus IL-3-treated pDCs is mostly driven by endogenous TNFα. Similarly, I found that also the expression of ISGs in IFNλ3 plus IL-3-treated pDCs is mostly driven by endogenous TNFα. Thus, data suggest that, in IFNλ3 plus IL-3-stimulated pDCs, endogenous TNFα is responsible for both the synergistic production of IFNα and the mRNA expression for ISGs.

Interestingly, ETA almost completely abrogated the expression of both IFNα and ISG mRNAs even in pDCs treated with either IFNλ3 or IL-3 (my unpublished observation). However, it was not possible to precisely quantify the effect of TNFα inhibitors on the production of IFNα by either IFNλ3-, or IL-3-, treated pDCs. In any case, ETA almost completely blocked IFIT1, ISG15 and MX1 mRNA expression in pDCs treated with IL-3, consistent with an inhibition on IFNα-dependent effects. A down-modulation of ISG expression by ETA was detected also in IFNλ3-treated pDCs, even though αIFNαR antibodies were ineffective under the same experimental conditions, thus excluding the involvement of IFNα. Although these results need to be carefully interpreted, it is possible that endogenous TNFα directly contributes to the transcriptional control of ISGs *via* NF-kB- and/or MAPK-dependent pathways, as proposed by the literature (183–185). In any case, the specificity of the effects by the TNFα inhibitors was demonstrated by their inability to significantly influence the prosurvival effects exerted by IFNλ3 and/or IL-3 in pDCs. Notably, combination of IFNλ3 and IL-3 did not produce a pDC prosurvival effect superior to that exerted by IFNλ3/IL-3 alone. Moreover, exogenous TNFα was found to directly induce both the production of low amounts of IFNα and expression of ISG15 mRNA, confirming pDC responsiveness to TNFα.

In sum, in this work I report that IFNλ3 and/or IL-3 induce, in human pDCs, the mRNA expression and production of both IFNα and TNFα, yet in a differentially regulated manner. As displayed in **Figure 17**, the combination of IL-3 and IFNλ3 induces a strong potentiation in the production of both TNFα and IFNα as compared to pDCs treated with IL-3/IFNλ3 alone. Synergistic increase of IFNα production by IFNλ3 plus IL-3-treated pDCs seems to be mostly dependent on endogenously produced TNFα (by approximately 80 %, panel 17C). By contrast, IFNα is produced in very low amounts by pDCs treated with each single agent (panels 17A and 17B), nonetheless IFNα mostly drives ISG mRNA expression in IL-3-treated pDCs (by approximately 60 %, panel 17B), but not in IFNλ3- (by 10 %, panel 17A) or IFNλ3 plus IL-3-treated pDCs (by 20 %, panel

17C). Even if IFNα is synergistically produced by IFNλ3 plus IL-3-treated pDCs, ISG mRNA expression under the latter experimental conditions derives in part from endogenously produced TNFα (by approximately 60 %, panel 17C), which also acts via IFNα, and in part from a direct effect of IFNλ3. Finally, endogenous TNFα drives ISG expression in both IL-3- (by approximately 80 %, panel 17B) and IFNλ3- (by approximately 40 %, panel 17A) stimulated pDCs.

All in all, data suggest that IFNλ3 and IL-3 may collaborate to induce some functional responses by pDCs at maximal levels. Specifically, pDCs take advantage of IL-3 because it potentiates their production of IFNα and their expression of ISG mRNA in response to type III IFNs (that are typical pDC activators). pDCs take also advantage of IFNλ3, since, on the other hand, it collaborates with IL-3 to synergistically increase the production of TNFα, that is important for pDC maturation. In this context, it is worth mentioning that IL-3 is often used in *in vitro* studies to maintain pDC survival in culture (41,42,172,173). This also occurs in experiments in which researchers investigate the effect on pDCs by a given stimulus. However, the potential contribution of IL-3 in determining the final results is almost always ignored (59, 172). As reported in this study, my data highlight that IL-3, in addition to prolong the survival of pDCs, may induce a number of responses that might sum up to those exerted by any stimulus under investigation. It derives that the potential contribution of IL-3, present in culture medium to incubate pDCs, should be always taken into consideration for a correct interpretation of final results.

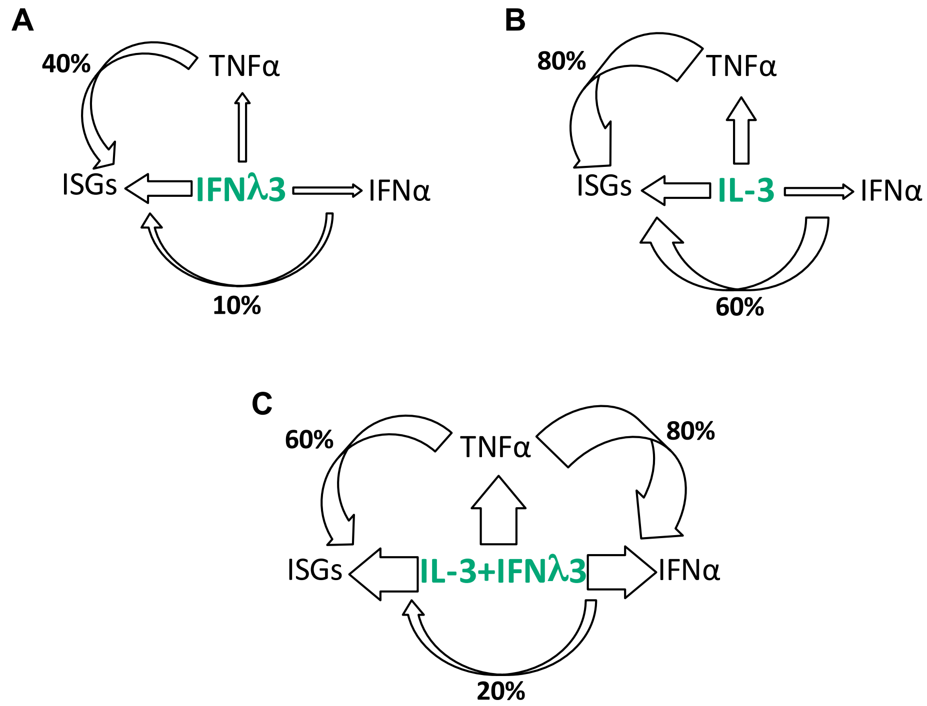


Figure 17. Schematic representation of the regulation of mRNA expression and cytokine production in human pDCs treated with IL-3 plus IFNλ3

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IV. ADDENDUM

CHARACTERIZATION OF TONSIL slan/MDC8^{+} cell FUNCTIONS AND PHENOTYPE

During my PhD, I have been also involved in a research project that focuses on the role in inflammation and cancer of a new subset of neglected subset of monocytes/myeloid dendritic cell, namely the slan (6-sulfo LacNAc^{+})/MDC8^{+} cells. Our group is in fact intensively working on these cells in collaboration with Prof. William Vermi (University of Brescia), recently demonstrating that they localize in metastatic tumor lymph nodes. However, despite the identification of these cells in a number of inflamed tissues (such as tonsils), detailed studies on phenotype and function of tissue slan/MDC8^{+} cells are still missing. Hence, in collaboration with my colleagues, I have performed experiments on tonsil slan/MDC8^{+} cells with the purpose of: i) comparing tonsil slan/MDC8^{+} cells phenotype and function with those of other known tonsil DC/macrophage populations; ii) clarifying the relationship between tonsil and blood slan/MDC8^{+} cells. Our data suggest that circulating slan/MDC8^{+} cells contribute to the pool of tonsil DCs and pave the way for a more detailed characterization of slan/MDC8^{+} cells in other pathological situations.

4.1 RESULTS (iii)

4.1.1 MATERIAL AND METHODS, RESULTS AND DISCUSSION ARE DESCRIBED IN THE FOLLOWING PUBLICATION

slan/M-DC8^{+} cells constitute a distinct subset of dendritic cells in human tonsils

slan/M-DC8$^+$ cells constitute a distinct subset of dendritic cells in human tonsils

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ABSTRACT

Human blood dendritic cells (DCs) include three main distinct subsets, namely the CD1c$^+$ and CD141$^+$ myeloid DCs (mDCs) and the CD303$^+$ plasmacytoid DCs (pDCs). More recently, a population of slan/M-DC8$^+$ cells, also known as "slanDCs", has been described in blood and detected even in inflamed secondary lymphoid organs and non-lymphoid tissues. Nevertheless, hallmarks of slan/M-DC8$^+$ cells in tissues are poorly defined. Herein, we report a detailed characterization of the phenotype and function of slan/M-DC8$^+$ cells present in human tonsils. We found that tonsil slan/M-DC8$^+$ cells represent a unique DC cell population, distinct from their circulating counterpart and also from all other tonsil DC and monocyte/macrophage subsets. Phenotypically, slan/M-DC8$^+$ cells in tonsils display a CD11c$^+$HLA-DR$^+$CD14$^+$CD11b$^{dim/neg}$CD16$^{dim/neg}$CX3CR1$^{dim/neg}$ marker repertoire, while functionally they exhibit an efficient antigen presentation capacity and a constitutive secretion of TNFα. Notably, such DC phenotype and functions are substantially reproduced by culturing blood slan/M-DC8$^+$ cells in tonsil-derived conditioned medium (TDCM), further supporting the hypothesis of a full DC-like differentiation program occurring within the tonsil microenvironment. Taken together, our data suggest that blood slan/M-DC8$^+$ cells are immediate precursors of a previously unrecognized competent DC subset in tonsils, and pave the way for further characterization of slan/M-DC8$^+$ cells in other tissues.

INTRODUCTION

Dendritic cells (DCs) represent a heterogeneous population of myeloid cells that are characterized by a very efficient capacity to present antigens to T cells. To date, three types of blood DCs, deriving from the same precursor [1], have been described in humans [2]. Specifically, the plasmacytoid DCs (pDCs), that are specialized in type I interferon production [3], and the conventional myeloid DCs (mDCs), that include the CD1c$^+$(BDCA1$^+$) DCs and the CD141$^+$(BDCA3$^+$) DCs, the latter ones being skilled at antigen cross-presentation to CD8$^+$ T cells [4]. All these DC populations have been also found in secondary lymphoid organs, including tonsils, spleen and lymph nodes [5-7]. An additional population of blood myeloid cells, that shares a number of phenotypic and functional characteristics with classical mDCs, has been described and called "slanDCs" by Schäkel and colleagues [8]. Accordingly, slanDCs have been identified by the use of a specific monoclonal antibody (M-DC8) recognizing the 6-Sulfo LacNAc (slan) carbohydrate modification of PSGL-1, whose acronym gave thus origin to the "slanDC" terminology [9-10]. However, on a two-dimensional flow cytometry dot plot of CD14 and CD16 expression in peripheral blood mononuclear cells (PBMCs), slan/M-DC8$^+$ cells in part overlap with CD14^{dim}CD16$^+$ monocytes [10-11], suggesting that they might actually represent a subset of non-classical monocytes [12-13]. Functionally,

blood slan/M-DC8+ cells have been described as potent pro-inflammatory cells based on their capacity to produce large amount of tumor necrosis factor alpha (TNFα) and IL-12p70 upon stimulation with toll-like receptor (TLR) ligands [10, 14]. Blood slan/M-DC8+ cells also promote proliferation, cytotoxicity and interferon-gamma (IFNg) production by natural killer (NK) cells [8, 15-16], and induce strong antigen-specific T-cell responses [9]. Furthermore, it is well established that slan/M-DC8+ cells locate in lymphoid and peripheral tissues, especially under inflammatory conditions. slan/M-DC8+ cells, in fact, have been identified in mucosal associated lymphoid tissue (such as tonsils [17],[11] and intestine Peyer's patches [17]), in skin of inflammatory diseases including lupus erythematosus [18] and psoriasis [14], in the colonic mucosa of Crohn disease patients [16-17], as well as in carcinoma-draining lymph nodes [11]. However, even though blood slan/M-DC8+ cell function and phenotype have been exhaustively delineated, an extensive comparison between blood and tissue slan/M-DC8+ cells, as well as between tissue slan/M-DC8+ cells and other tissue DC/macrophage populations, has never been performed.

In this study, we have performed a detailed characterization of slan/M-DC8+ cells in tonsils, in turn demonstrating that they represent a unique DC population, clearly different from any other tonsil DC or monocyte/macrophage population described to date [19]. Moreover, our data suggest that blood slan/M-DC8+ cells contribute to replenish such slan/M-DC8+ DC pool in tonsils, thus uncovering new information on plasticity by blood slan/M-DC8+ cells and their ultimate commitment within tissue microenvironments.

RESULTS

slan/M-DC8+ cells as a unique DC population in human tonsils

To better characterize the frequency, phenotype, differentiation state and function of slan/M-DC8+ cells in tissues, we initially analyzed, by flow cytometry, single cell suspensions from a large set of human tonsils. All tonsil samples were obtained from children affected by recurrent, chronic tonsillitis. Using the gating strategy illustrated in Supplementary Figure S1, among HLA-DR+CD11c+ myeloid cells we could identify two DC populations, namely the CD1c+(BDCA-1+) DCs and the CD141+(BDCA-3+) DCs (Figure 1a), as previously reported by others [19], and a CD14+CD11b+ monocyte/macrophage population. In addition, we could also identify the slan/M-DC8+ cells (Figure 1a). We calculated that the slan/M-DC8+ cells account for about 0.1 % of the total CD45+ leukocytes (data not shown), and about 10 % of the total HLA-DR+CD11c+ myeloid cells in tonsils (Figure 1b). In such regard, slan/M-DC8+ cell frequency was found similar to that of CD141+ DCs (8.1 ± 3.1 %; n = 22), but consistently lower than those of CD1c+ DCs (29.2 ± 13.5 %; n = 21) or CD14+CD11b+ monocytes/macrophages (16.3 ± 13 %; n = 15) (Figure 1b). As assessed by cytospin preparations of sorted cells, tonsil slan/M-DC8+ cells displayed a typical DC shape, similar to CD1c+ and CD141+ DCs, yet showing a larger size (Figure 1c). Conversely, CD14+CD11b+ monocytes/macrophages consist of a heterogeneous population that includes large cells with typical macrophage morphology, containing phagocytic vacuoles admixed to smaller cells with round morphology and similar to monocytes (Figure 1c). Among the different tonsil compartments identified by the BCL6/CKP staining (Figure 2a), slan/M-DC8+ cells were found mainly located in the crypts (Figure 2b), as previously reported [11], while CD14+CD11b+ monocytes/macrophages were predominant in the inter-follicular (IF) area (Figure 2c).

By characterizing their phenotype by flow cytometry, we observed that, despite donor variability, and in contrast to their blood counterpart, tonsil slan/M-DC8+ cells did express CD14, a feature shared with monocytes/macrophages (Figures 1d and 2d). By contrast, CD11b was found neither in slan/M-DC8+ cells, nor in other DCs (Figures 1e and 2d). Moreover, by IHC staining of tonsil sections, the anti-CD11b antibody strongly stained follicular DCs (Figure 2e), neutrophils (Figure 2f) and a population of small mononuclear cells (likely monocytes, Figure 2g), but not slan/M-DC8+ cells (Figure 2g). A weak CD11b reactivity was also observed in larger CD14+ mononuclear cell in the IF area (Figure 2c), therefore accounting for the CD11b+CD14+ population detectable by flow cytometry (Figure 1a).

The possibility that tonsil slan/M-DC8+ cells might overlap with a recently identified population of CD14+FcεRI+ present in human inflammatory fluids, and able to induce Th17 differentiation [20], was also excluded since tonsil slan/M-DC8+ cells do not express FcεRI (Figure 1f). Interestingly, we could observe that FcεRI is, however, expressed by tonsil CD1c+ DCs (Figure 1f), which are instead CD14-negative (Figures 1d and 2d). By flow cytometry, we found that CD163, previously reported as a marker for axillary lymph node CD14+ cells [7], was variably expressed by all cell populations under investigation (Figure 1g). Finally, analysis of costimulatory molecule expression revealed that, while CD86 was expressed in slan/M-DC8+ cells, mDCs and CD11b+CD14+ monocytes/macrophages (Figure 1h), CD83 was regularly absent in all these cell populations (Figure 1i). Notably, both CD40 and CD80 were expressed at the highest levels in tonsil slan/M-DC8+ cells (Figure 1j, 1k). Finally, we found that tonsil slan/M-DC8+ cells do not express CD206 and CD209 (data not shown). Altogether, these data qualify tonsil slan/M-DC8+ cells as

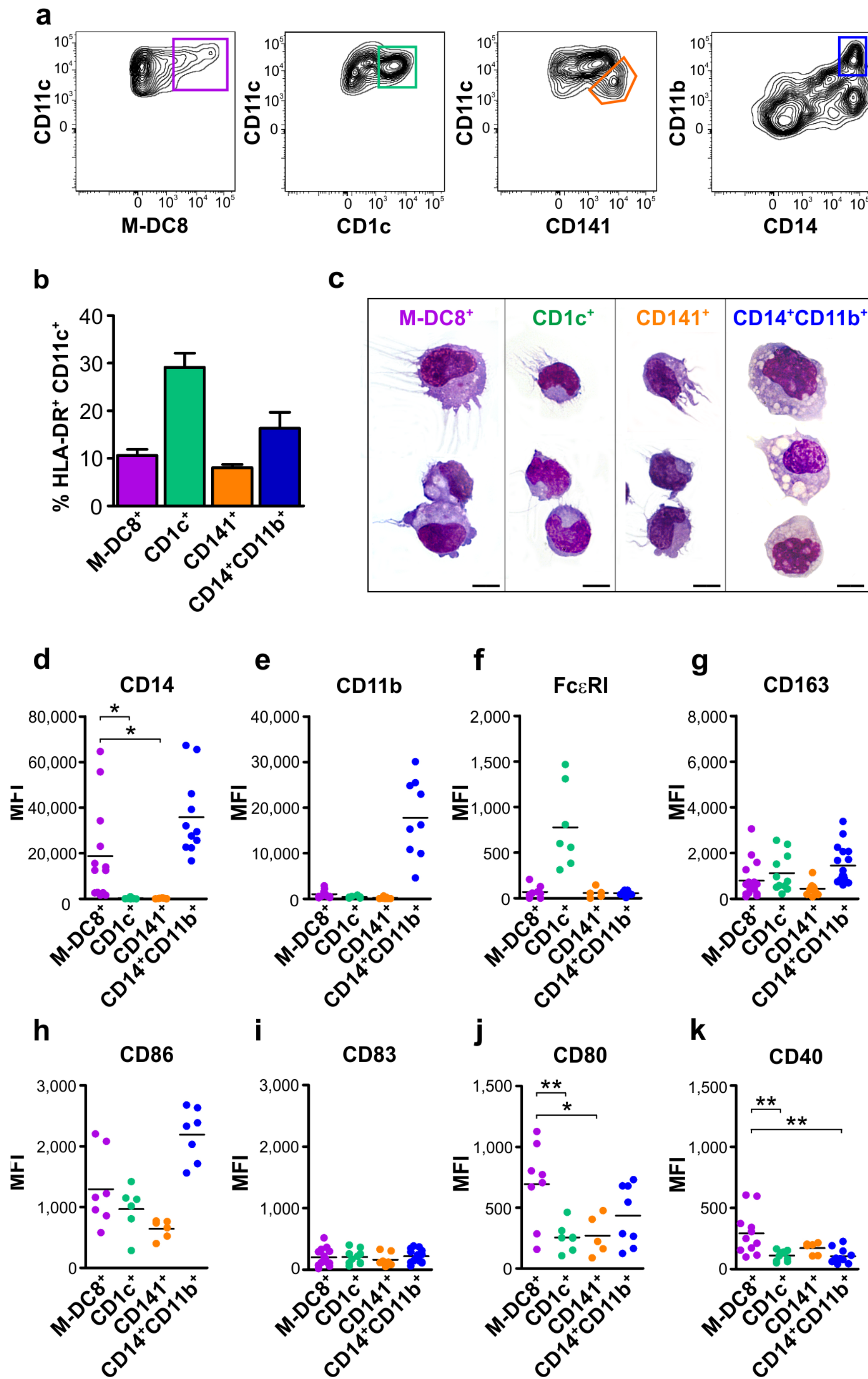

Figure 1: Phenotypic characterization of slan/M-DC8⁺ DCs and other myeloid populations in human tonsils. a. Contour plots illustrate how slan/M-DC8⁺ DCs, as well as CD1c⁺ DCs, CD141⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages, were identified within tonsil cell suspensions by flow cytometry (a more complete and detailed gating strategy is reported in Supplementary Figure S1). **b.** Graph shows the percentages of tonsil slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages among all HLA-DR⁺CD11c⁺ myeloid cells (n = 15-20). **c.** Morphology of sorted slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages on cytospins stained by May-Grunwald Giemsa (scale bar = 20 μm). **d.-k.** Graphs show the expression levels of each indicated marker in tonsil slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages, as measured by flow cytometry. Values indicate the mean fluorescence intensity (MFI) for each sample. $*P < 0.05$; $**P < 0.01$, by one-way ANOVA test.

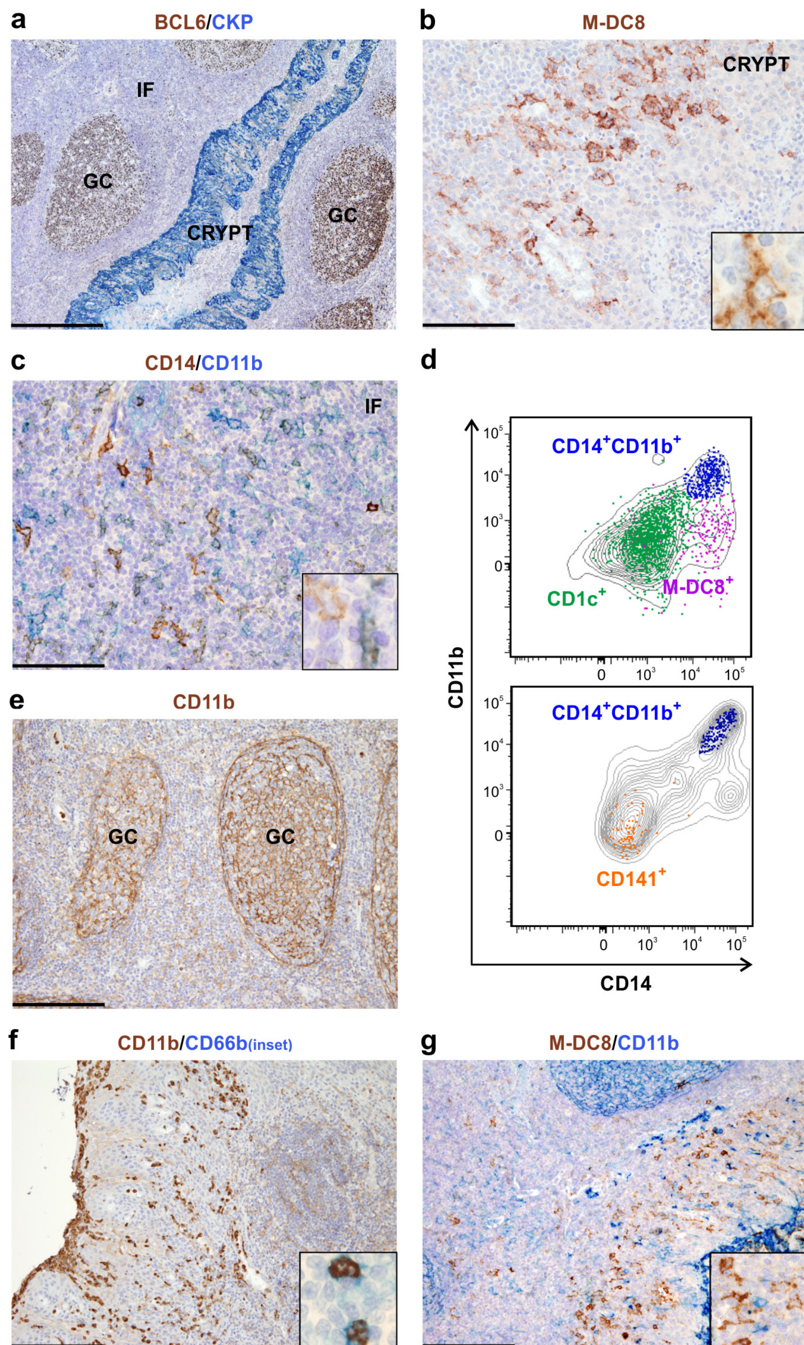

Figure 2: slan/M-DC8⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages are distinct cell populations in human tonsils. **a.-c.**; **e.-g.** Sections are from tonsil samples and stained as indicated by labels. **a**. Pan-cytokeratin (CKP) and BCL6 identify different compartments including follicles with BCL6⁺ germinal centre (GC) B-cells, CKP⁺ epithelial crypts and the interfollicular area (IF) between two or more follicles. **b.** High power view of a tonsil crypt area showing slan/M-DC8⁺ DCs intermingled with epithelial cells. Inset shows a higher magnification of slan/M-DC8⁺ DC morphology. **c.** High power view of an interfollicular area showing a CD14/CD11b double staining. Inset shows a higher magnification of a CD14⁺ cell as well as a CD14⁺CD11b⁺ cell. **e.**, **f.** CD11b stains both follicular DCs in germinal centers (e), and CD66b⁺ neutrophils in the tonsil epithelium (f); inset in panel f shows a high power view of CD11b⁺CD66b⁺ neutrophils. (**g**. and inset) Tonsil slan/M-DC8⁺ DCs are instead completely negative for CD11b. Sections are counterstained with Meyer's haematoxylin. Original magnifications: 40X (panel a, scale bar 500 μm); 100X (panels e-g, scale bar 200 μm); 200X (panels b,c, scale bar 100 μm); 600X (insets). **d.** Overlay plots displaying the CD11b and CD14 levels in tonsil slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs and CD14⁺CD11b⁺ monocytes/macrophages, as measured by flow cytometry. Single cell populations were first identified by specific markers (as depicted in Figure 1a) and then overlaid on the contour plots of total CD11c⁺HLA-DR⁺ cells. A representative experiment, out of at least 4 performed with similar results, is shown.

a distinct DC population. Data also suggest that, by flow cytometry, CD11b could be a much more useful marker to distinguish tonsil CD11b$^{dim/neg}$ DC subsets from tonsil CD11b^{bright} monocytes/macrophages than the commonly used CD14 or CD163.

Blood slan/M-DC8$^+$ cells incubated in tonsil-derived conditioned medium (TDCM) acquire the phenotype of tonsil slan/M-DC8$^+$ DCs

A comparative analysis between blood versus tonsil slan/M-DC8$^+$ cells revealed substantial differences in morphology and phenotype. In fact, blood slan/M-DC8$^+$ cells are round with irregularly shaped nucleus (Figure 3a), while slan/M-DC8$^+$ DCs purified from tonsils are larger cells with large round nuclei and acquire dendrites (Figures 1c and 3c). Phenotypically, blood and tonsil slan/M-DC8$^+$ cells are CD83-negative and maintain equivalent levels of M-DC8 (Figure 3b, 3d). By contrast, tonsil slan/M-DC8$^+$ DCs express lower levels of both CD16 and CX3CR1, but higher levels of HLA-DR, CD11c and CD14 than blood slan/M-DC8$^+$ cells (Figure 3b, 3d), thus suggesting that the latter cells modify their phenotype once recruited into tonsils.

Concomitantly with the analysis of *ex vivo* isolated tonsil slan/M-DC8$^+$ DCs, we set up an *in vitro* model aimed at inducing a tonsil-like phenotype in slan/M-DC8$^+$ cells purified from the blood of healthy donors. Specifically, we generated various TDCMs and used them as a culture medium for blood slan/M-DC8$^+$ cells. As shown in Figure 3e, blood slan/M-DC8$^+$ cells conditioned by TDCM for 5 days become morphologically very similar to slan/M-DC8$^+$ DCs directly purified from tonsils (Figure 3c). We also observed that TDCM-conditioned slan/M-DC8$^+$ cells down-modulated CD16 and CX3CR1, while they up-regulated HLA-DR, CD11c and CD14 (Figure 3f), thus mirroring the phenotype of freshly purified tonsil slan/M-DC8$^+$ DCs (Figure 3d). Accordingly, CD83 remained negative also in TDCM-conditioned slan/M-DC8$^+$ cells (Figure 3f). Taken together, these experiments demonstrate that TDCM substantially induces a tonsil-like phenotype in blood slan/M-DC8$^+$ cells, thus supporting the hypothesis of a “differentiation program” that peripheral slan/M-DC8$^+$ cells undertake upon their arrival in tonsils.

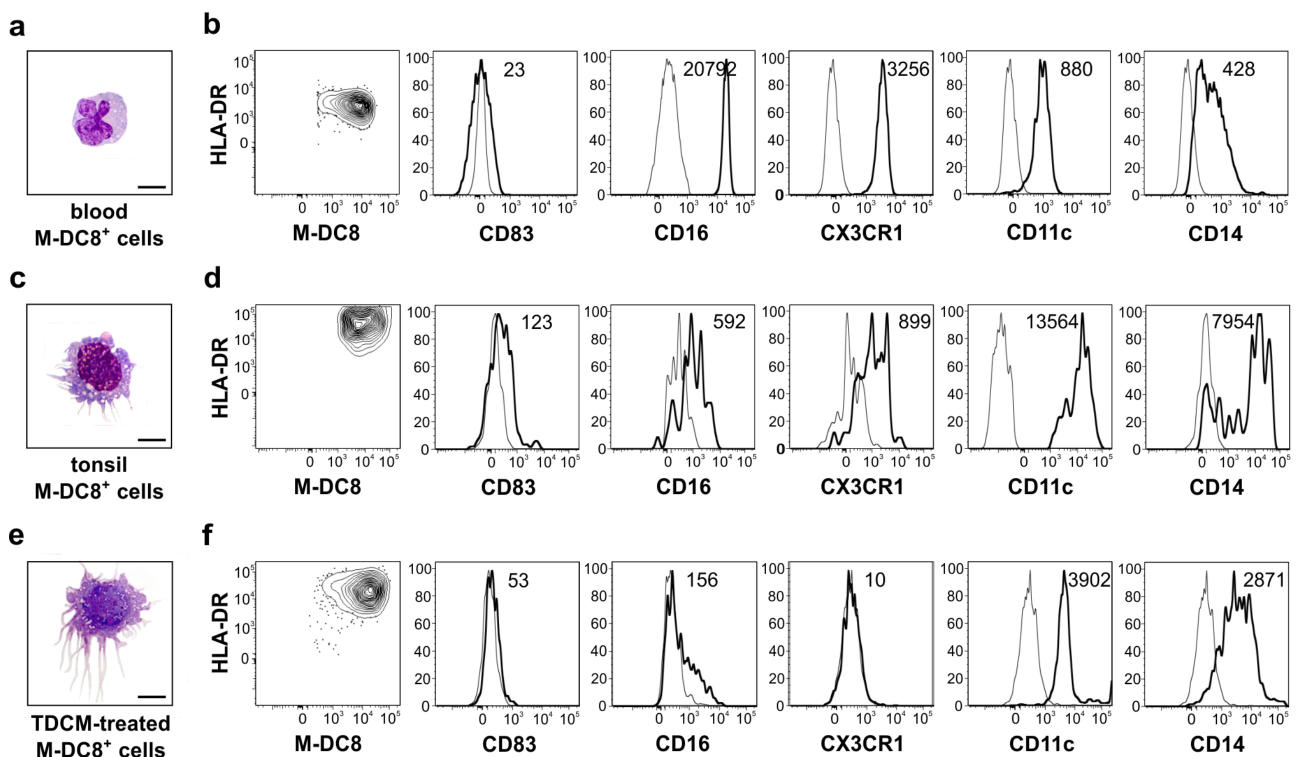

Figure 3: Blood slan/M-DC8$^+$ cells incubated in tonsil derived-conditioned medium (TDCM) acquire the morphology and phenotype of tonsil slan/M-DC8$^+$ DCs. Blood slan/M-DC8$^+$ cells were cultured for 5 d in TDCM and then compared to tonsil slan/M-DC8$^+$ DCs in terms of morphology and phenotype. Morphology of, respectively, blood **a.**, tonsil-sorted **c.**, and TDCM-conditioned **e.**, slan/M-DC8$^+$ cells on cytospins stained by May-Grunwald Giemsa (scale bar = 20 µm) is shown. **b.**, **d.**, **f.** Contour plots and histograms illustrating the expression of each indicated marker (thick black line) versus related isotype control (thin black line) in blood **b.**, tonsil-sorted **d.** and TDCM-conditioned **f.** slan/M-DC8$^+$ cells. MFI value for each marker is also reported in corresponding histogram. A representative experiment out of 4 performed with similar results is shown.

Blood slan/M-DC8$^+$ cells exhibit a remarkable plasticity

In subsequent experiments, we compared the phenotype of TDCM-conditioned slan/M-DC8$^+$ cells with the phenotypes acquired by blood slan/M-DC8$^+$ cells incubated for 5 days in the presence of either GM-CSF plus IL-4, which is known to generate competent DCs from circulating slan/M-DC8$^+$ cells [21], or IL-34, which induces a macrophage differentiation from classical CD14$^+$ monocytes [22]. Notably, blood slan/M-DC8$^+$ cells express the highest levels of CD115/CSF1R (e.g., the receptor shared by both M-CSF and IL-34) as compared to the other blood DC and monocyte subsets (Figure 4a). First of all, we found that, unlike control medium, all stimulatory conditions maintained the survival of slan/M-DC8$^+$ cells at variable levels (Figure 4b). Then, we observed that culturing slan/M-DC8$^+$ cells with GM-CSF plus IL-4, IL-34 or TDCM, significantly up-regulated the expression of HLA-DR, in line with an *in vitro*-induced differentiation process (Figure 4c). A similar trend was also observed for the expression of CD11c (Figure 4d), even though its modulation did not reach statistical significance. Interestingly, surface CD163, CD14 and CD16, which are typically co-expressed by macrophages [22-23], were either upregulated (CD163 and CD14) or maintained (CD16) in IL-34-treated slan/M-DC8$^+$ cells (Figure 4e-4g). Conversely, the same three markers were almost negative when slan/M-DC8$^+$ cells where cultured in GM-CSF plus IL-4 (Figure 4e-4g), in line with their DC-like differentiation [21, 24]. In such regard, TDCM-conditioned slan/M-DC8$^+$ cells, as GM-CSF plus IL-4-conditioned slan/M-DC8$^+$ cells, did express either CD163 or CD16 at minimal levels (Figure 4e, 4g). Finally, TDCM-conditioned slan/M-DC8$^+$ cells were found to express moderate amounts of CD14 (Figure 4f), yet at significantly higher levels than their blood counterpart ($P < 0.001$ by two-tailed unpaired t test), consistent with the CD14 detection in tonsil slan/M-DC8$^+$ DCs (Figure 1d). Our data demonstrate that TDCM-conditioned slan/M-DC8$^+$ cells display a DC-like antigen expression profile that is more similar to that acquired by GM-CSF plus IL-4-conditioned slan/M-DC8$^+$ cells than to the macrophage-like one induced by IL-34. Interestingly, we found that GM-SCF, but not IL-4, was detectable in all TDMCs used for our *in vitro* differentiation (ranging from 200 pg ml^{-1} to 2800 pg ml^{-1}). Taken together, data also uncover that blood slan/M-DC8$^+$ cells exhibit a remarkable plasticity and differentiate into either DCs or macrophages, depending on the type of differentiation factors they are exposed to.

Tonsil slan/M-DC8$^+$ DCs efficiently present antigens to T cells

Extending previous observations [11], double stains for M-DC8 and CD3, CD4 or CD8 (Figure 5a-5c) confirmed that, in human tonsils, slan/M-DC8$^+$ DCs interact with T cells. In addition, some CD3$^+$ T cells contacting slan/M-DC8$^+$ DCs also co-stain for the proliferating marker Ki67 (Figure 5d). Based on these findings, we then analyzed the Ag presentation capacity by slan/M-DC8$^+$ DCs isolated from tonsils. We thus sorted CD11c$^+$slan/M-DC8$^+$ DCs along with all other DC/macrophage populations and then cultured each cell type with allogeneic CD4$^+$ T lymphocytes to measure their proliferation after 7 days (Figure 5e, showing a representative experiment). We observed that, at least at their highest concentrations, tonsil slan/M-DC8$^+$ DCs displayed, similarly to CD1c$^+$ or CD141$^+$ DCs, an Ag presentation capacity significantly higher than tonsil CD14$^+$CD11b$^+$ monocytes/macrophages. The latter cells, indeed, were reproducibly found to be very poor stimulatory APCs for T cells (Figure 5e, 5f).

Subsequently, we analyzed the capacity of TDCM-conditioned slan/M-DC8$^+$ cells to perform Ag presentation under autologous settings. We thus co-cultured blood and TDCM-conditioned slan/M-DC8$^+$ cells with autologous CD4$^+$ T cells for 7 d in the presence of Tetanus Toxoid (TT). We observed that TDCM-conditioned slan/M-DC8$^+$ cells induced a CD4$^+$ T cell proliferation at higher extent than freshly isolated, autologous blood slan/M-DC8$^+$ cells, while peripheral CD14$^+$ monocytes (either freshly isolated or conditioned with TDCM) resulted to be poor APCs (Figure 5g). Donor-matched blood CD1c$^+$ DCs performed the strongest Ag presentation capacity without the necessity to differentiate. Indeed, freshly isolated as well as TDCM-conditioned CD1c$^+$ DCs promoted an equivalent T cell proliferation (Figure 5g). Of note, the Ag presentation capacity by TDCM-conditioned slan/M-DC8$^+$ cells (Figure 5g) and freshly purified tonsil slan/M-DC8$^+$ DCs cultured at the same concentration (e.g., 5,000 APCs) were similar (Figure 5f). Taken together, data support the notion that tonsil slan/M-DC8$^+$ DCs represent an additional bona fide DC subset present in tonsils. Data also demonstrate that TDCM could be used as a valid *in vitro* model to induce, starting from blood slan/M-DC8$^+$ cells, not only the phenotype but also the APC function of tonsil slan/M-DC8$^+$ DCs.

Tonsil slan/M-DC8$^+$ DCs constitutively secrete TNFα but not IL-12p70

Finally, we analyzed the capacity of tonsil slan/M-DC8$^+$ DCs to produce TNFα and IL-12p70. CD1c$^+$ DCs, CD14$^+$CD11b$^+$ monocytes/macrophages and CD303$^+$ pDCs were also tested for comparison purposes. Initially,

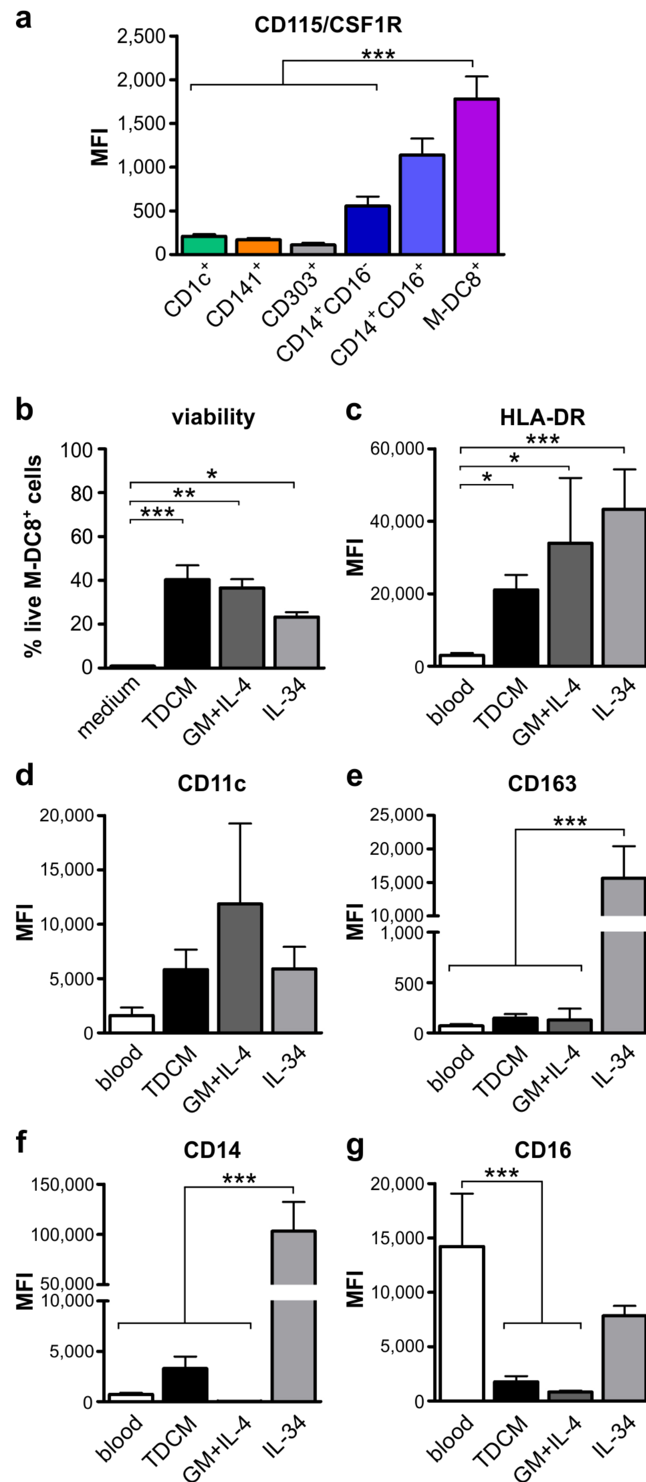

Figure 4: Blood slan/M-DC8$^+$ cells display the capacity to polarize toward either a "DC-like" or a "macrophage-like" phenotype. a. Expression levels of CD115/CSF1R in blood CD1c$^+$, CD141$^+$, CD303$^+$, CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and slan/M-DC8$^+$ cells within freshly isolated peripheral PBMCs (n = 4). **b.-g.** Blood slan/M-DC8$^+$ cells were cultured for 5 days in: medium alone (only in panel b), tonsil-derived conditioned medium (TDCM), 50 ng ml^{-1} GM-CSF plus 20 ng ml^{-1} IL-4, or 100 ng ml^{-1} IL-34. **b.** Graph shows the percentage of live slan/M-DC8$^+$ cells after a 5 d-incubation under each stimulatory condition (n = 8-10). Cell viability was established by flow cytometry, using Vybrant® DyeCycle™ Violet Stain. Live cells were gated (e.g., Vybrant negative slan/M-DC8$^+$ cells) and surface marker expression then analyzed. **c.-g.** Graphs show the levels of expression of HLA-DR (c), CD11c (d), CD163 (e), CD14 (f) and CD16 (g) in 5 d-treated slan/M-DC8$^+$ cells and freshly purified blood slan/M-DC8$^+$ cells (n = 8-15). *P < 0.05; **P < 0.01; ***P < 0.001, by one-way ANOVA test.

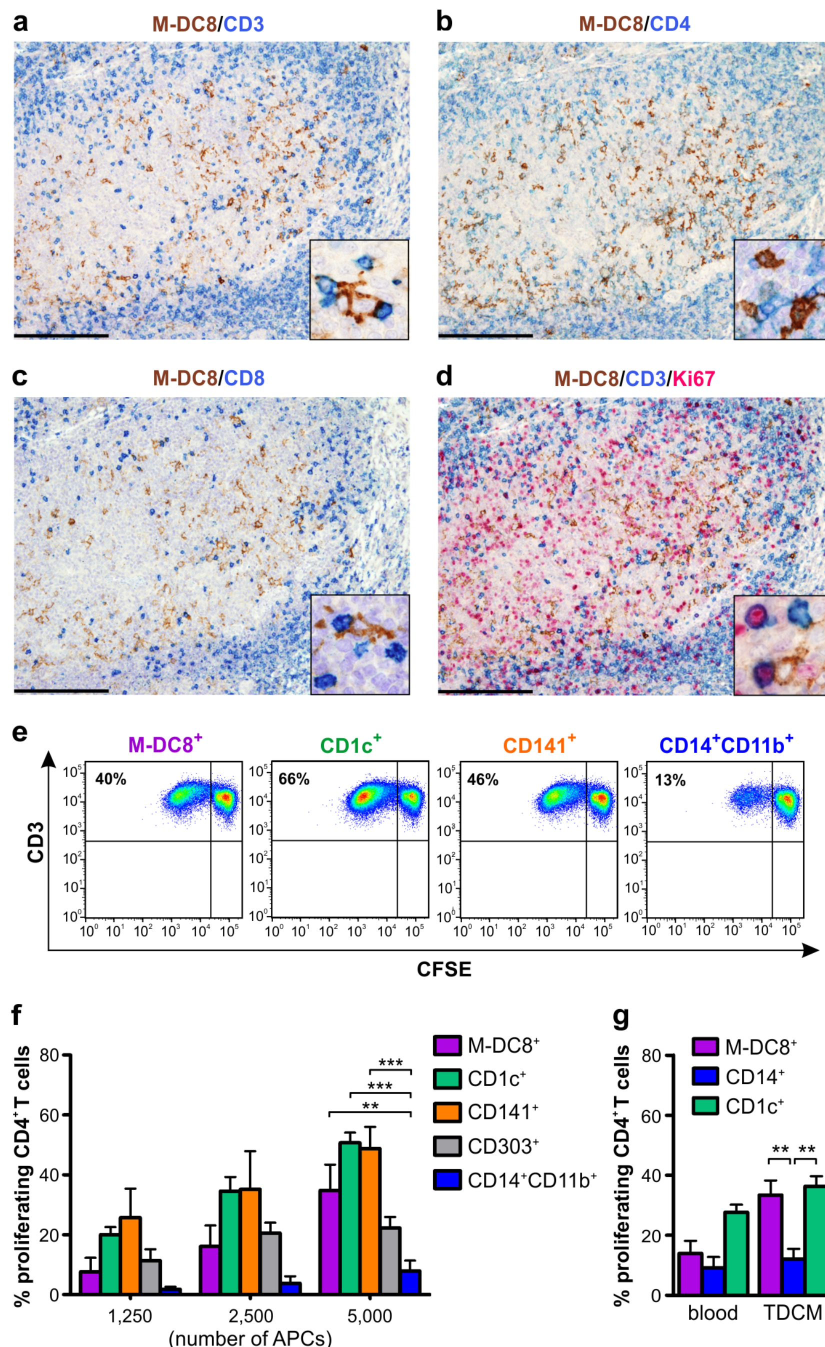

Figure 5: slan/M-DC8⁺ DCs interact with T cells in tonsils and display a remarkable antigen presentation capacity. **a.-d.** Sections are from human tonsils stained as indicated by labels. Double staining shows that a fraction of slan/M-DC8⁺ DCs interact with CD3⁺ T cells in the crypt (a), which are either CD4⁺ (as dominant population, b) and CD8⁺ (c). **d**. Triple staining shows that the T cell population interacting with slan/M-DC8⁺ DCs includes a fraction of CD3⁺Ki67⁺ proliferating T lymphocytes. Cell interactions are illustrated by high power view insets in panels a-d. Sections are counterstained with Meyer's haematoxylin. Original magnifications: 100X (panels a-d, scale bar 200 µm); 600X (insets in a-d). **e.**, **f.** Sorted tonsil slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs, CD14⁺CD11b⁺ monocytes/macrophages and CD303⁺ pDCs were co-cultured with CFSE-labeled allogeneic CD4⁺ T cells for 7 days. T cell proliferation was then determined by the CFSE dilution method. **e.** Representative experiment displaying T cell proliferation by the CSFE assay, in which $5x10^4$ T cells were cultured with $5x10^3$ cells of sorted tonsil slan/M-DC8⁺ DCs, CD1c⁺ DCs, CD141⁺ DCs, CD14⁺CD11b⁺ monocytes/macrophages in a final volume of 200 µL. **f.** Graph shows the % of T cell proliferation induced by an increasing number of each tonsil cell population, as indicated (n = 3-7). $**P < 0.01$; $***P < 0.001$, by two-way ANOVA test. **g.** $5x10^3$ freshly isolated (blood) or 5-d TDCM-conditioned slan/M-DC8⁺ cells, CD14⁺ monocytes or CD1c⁺ DCs from the same healthy donors were co-cultured with $5x10^4$ CFSE-labeled autologous CD4⁺ T cells in the presence of TT antigen in a final volume of 200 µL, for 7 days. Graph shows the % of T cell proliferation induced by each cell population (n = 3-7). $**P < 0.01$, by two-way ANOVA test.

we took advantage of a cytokine secretion assay [11], since it allows the direct analysis of cytokine secretion at a single-cell level within a heterogeneous cell population. We found that tonsil slan/M-DC8+ DCs constitutively secrete TNFα (Figure 6a and Supplementary Figure S2, this latter showing one representative experiment), unlike blood slan/M-DC8+ cells [11]. A constitutive TNFα production was also observed in CD1c+ DCs (Figure 6b) and, at higher levels, in CD14+CD11b+ monocytes/macrophages (Figure 6c), but not in CD303+ pDCs (Figure 6d). Stimulation with TLR agonists in combination with IFNγ slightly increased TNFα secretion in slan/M-DC8+ DCs, CD1c+ DCs and CD14+CD11b+ monocytes/macrophages (Figure 6a-6c). An induction of TNFα

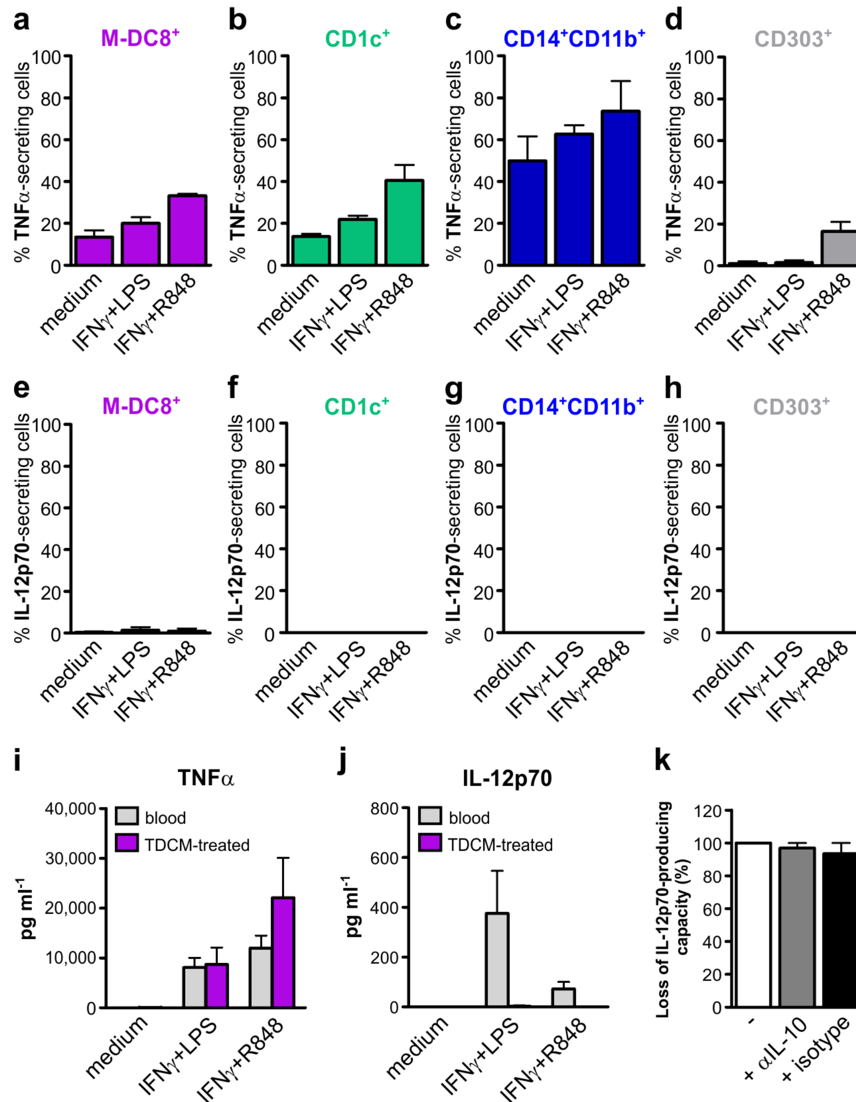

Figure 6: Tonsil slan/M-DC8+ DCs, CD1c+ DCs, CD14+CD11b+ monocytes/macrophages and CD303+ pDCs produce TNFα but not IL-12p70. **a.-h.** Tonsil cell suspensions were incubated with or without 100 U ml^{-1} IFNγ plus either 100 ng ml^{-1} LPS or 5 µM R848, either for 4 h (to detect TNFα secretion, a-d), or for 12 h, after a 6 h pre-incubation (to detect IL-12p70 secretion, e-h). Graphs show TNFα-secreting slan/M-DC8+ DCs **a.**, CD1c+ DCs **b.**, CD14+CD11b+ monocytes/macrophages **c.** and CD303+ pDCs **d.**, or IL-12p70-secreting slan/M-DC8+ DCs **e.**, CD1c+ DCs **f.**, CD14+CD11b+ monocytes/macrophages **g.** and CD303+ pDCs **h.**. The graphs show the mean of cytokine secreting cells (as percentage of each cell population) calculated from 4 experiments. **i.**, **j.** 2.5x10^4 100 µl^{-1} blood (gray bars), or 5-d TDCM-conditioned (purple bars), slan/M-DC8+ cells were incubated for 24 h with or without 100 U ml^{-1} IFNγ plus either 100 ng ml^{-1} LPS or 5 µM R848 to measure the levels of TNFα (i) and IL-12p70 (j) in cell free supernatants by ELISA (*n* = 5-7). **k.** Blood slan/M-DC8+ cells were either immediately stimulated with 100 U ml^{-1} IFNγ plus 100 ng ml^{-1} LPS for 24 h, or conditioned in TDCM, in the presence or absence of 10 µg ml^{-1} anti-IL-10 or IgG2a isotype control mAbs. After 5 d of incubation, TDCM-conditioned slan/M-DC8+ cells were stimulated with IFNγ plus LPS for 24 h. IL-12p70 was then measured in cell-free supernatants by ELISA. Graph show the loss of IL-12p70-producing capacity (in %) by TDCM-conditioned slan/M-DC8+ cells after IFNγ plus LPS stimulation (in the absence or the presence of neutralizing mAbs), as compared to blood slan/M-DC8+ cells incubated with IFNγ plus LPS.

production in CD303$^+$ pDCs was instead observed only after R848 stimulation (Figure 6d), consistent with the absence of TLR4 expression by these cells. By contrast, no IL-12p70 secretion could be detected either by tonsil slan/M-DC8$^+$ DCs cells, or by the other cell populations, under any experimental condition used (Figure 6e-6h).

Such an *ex vivo* analysis on tonsil slan/M-DC8$^+$ DCs was further supported by *in vitro* data using the TDCM-differentiation model. In fact, while blood slan/M-DC8$^+$ cells incubated for 24 h with IFNγ plus either LPS or R848 produced both TNFα and IL-12p70 (by ELISA) (Figure 6i, 6j; grey bars), TDCM-conditioned slan/M-DC8$^+$ cells retained the capacity to produce only TNFα but not IL-12p70 (Figure 6i, 6j; purple bars).

Given the ability of IL-10 in inhibiting the production of IL-12p70 by monocyte-derived DCs [25-26], we then analyzed whether IL-10 was contained in TDCMs, finding remarkable levels of it (244 ± 179 pg ml^{-1}; $n = 9$) in all TDCMs. To clarify whether TDCM-derived IL-10 might be responsible for the loss of IL-12p70 production capacity by activated slan/M-DC8$^+$ cells, we therefore added an anti-IL-10 neutralizing antibody to blood slan/M-DC8$^+$ cells incubated with TDCM. Then, after 5 d of differentiation, we re-stimulated the cells with IFNγ plus LPS, in the presence of anti-IL-10 neutralizing antibody or its related isotype control, for additional 24 h. As shown in Figure 6k, the inability to produce IL-12p70 by TDCM-conditioned slan/M-DC8$^+$ cells under conditions in which IL-10 is neutralized remained unchanged. In control experiments, the same antibody completely restored the IL12p70 production abrogated by exogenous IL-10 (data not shown).

DISCUSSION

In this study, we have performed an extensive phenotypic and functional characterization of slan/M-DC8$^+$ cells in human tonsils, which ultimately proves that these cells represent a unique CD11c$^+$HLA-DR$^+$CD14$^+$CD11b$^{dim/neg}$CD16$^{dim/neg}$CX3CR1$^{dim/neg}$ population of DCs, different from other classical CD1c$^+$ and CD141$^+$ mDCs or CD14$^+$CD11b$^+$ monocytes/macrophages. Our data also demonstrate that tonsil slan/M-DC8$^+$ DCs differ from their blood counterparts, characterized by a CD11c$^+$HLA-DR$^+$CD14^{dim}CD11b^{dim}CD16^{bright}CX3CR1^{bright} phenotype, suggesting that blood slan/M-DC8$^+$ cells undergo a DC differentiation process once migrated into tonsils. Functionally, tonsil slan/M-DC8$^+$ DCs proved to be competent in antigen presentation and to constitutively produce TNFα. Moreover, blood slan/M-DC8$^+$ cells incubated with TDCM for 5 days were found to acquire a tonsil-like slan/M-DC8$^+$ DC phenotype and function, suggesting the involvement of soluble factors produced by the tonsil environment for such a differentiation process.

slan/M-DC8$^+$ cells are usually CD14^{dim} in different compartments (e.g., blood or skin [9, 27]). In this study, an unexpected observation that we uncovered is that, unlike their blood counterpart, tonsil slan/M-DC8$^+$ DCs express CD14 at variable but significant levels. This finding is particularly interesting since tissue CD14$^+$ myeloid cells are conventionally limited to macrophages (e.g., in human skin [28], tonsils [19], lymph nodes [7], intestine [29] and spleen [30]). Nevertheless, a subset of CD1c$^+$FcεRI$^+$ inflammatory DCs has been recently reported to express CD14 [20]. Another study also reported the existence of CD14$^+$CD163^{dim}M-DC8$^+$ cells in intestinal lamina propria, displaying features of both macrophages and DCs [31]. Interestingly, this population share, at least in part, the DC phenotype of tonsil slan/M-DC8$^+$ DCs herein described. Moreover, a very recent study shows that CD172a$^+$ slanDCs in Crohn's disease tissues express CD14 [32]. Taken together, all these findings indicate that the expression of CD14 is not specific for tissue macrophages since it can be also shared by some DC subsets in tissues. By contrast, CD11b was found highly expressed in tonsil CD14$^+$ cells but not in slan/M-DC8$^+$ DCs or in all other DC populations, suggesting that, at least in tonsils, surface CD11b might better discriminate between DCs and monocytes/macrophages.

In this study, tonsil DCs, including slan/M-DC8$^+$ DCs, were found negative for CD83, confirming a previous observation [19]. However, tonsil slan/M-DC8$^+$ DCs do express other costimulatory molecules, such as CD40, CD80 and CD86. Moreover, tonsil slan/M-DC8$^+$ DCs were found to display a proficient Ag presentation capacity, significantly higher than tonsil CD14$^+$CD11b$^+$ monocytes/macrophages and similar to other DCs. Thus, despite tonsil M-DC8$^+$ cells have been already defined as DCs simply based on their morphology and localization [17], herein we provide the first direct demonstration of their remarkable antigen presentation capacity. Furthermore, our findings are consistent with previous *in vitro* data demonstrating a superior Ag presentation capacity by blood slan/M-DC8$^+$ cells than CD14$^+$ monocytes [8], as well as a stronger priming activity for naïve T cells by GM-CSF plus IL-4-treated slan/M-DC8$^+$ cells than GM-CSF plus IL-4-treated CD14$^+$M-DC8$^-$ cells [21].

As mentioned, we also show that blood and tonsil slan/M-DC8$^+$ cells display a substantially different phenotype. We believe that this is strictly coupled with the slan/M-DC8$^+$ cell migration into tonsils and terminal differentiation into DCs. This is also in accordance with the previous demonstration that *in vitro* cultured blood slan/M-DC8$^+$ cells, once detaching from erythrocytes (a process mimicking the exit from the vessels), rapidly acquire several characteristics of DCs [8, 10]. Moreover, it has been already reported that CD16$^+$ monocytes (which include slan/M-DC8$^+$ cells), but not CD14$^+$CD16$^-$ monocytes, preferentially become DCs in a model of reverse transmigration through endothelial cells [33].

Notably, we also found that tonsil slan/M-DC8$^+$ DCs dramatically down-regulate their CD16 expression, which was speculated to represent a step required to differentiate into DCs [21]. Finally, tonsil slan/M-DC8$^+$ DCs also down-modulate CX3CR1 expression, a phenomenon that might be caused by its internalization after binding with CX3CL1/Fractalkine, its ligand, which is highly expressed in the crypts of inflamed tonsils [34], where slan/M-DC8$^+$ DCs frequently localize [11, 17].

Previous studies have highlighted the proinflammatory nature of circulating slan/M-DC8$^+$ cells, for their capacity to produce high levels of TNFα and, particularly, IL-12p70 [9-10], in response to TLR ligands [14]. Immunofluorescence staining of skin lesions from cutaneous lupus erythematosus and psoriasis patients has confirmed that slan/M-DC8$^+$ cells are TNFα-positive also in tissues [14, 18]. TNFα expression in colonic mucosa-associated slan/M-DC8$^+$ cells of Chron's disease patients has also been reported [32]. Herein, we show a constitutive secretion of TNFα by a fraction of slan/M-DC8$^+$ DCs within tonsil cell suspensions, which was also observed to occur in the case of CD1c$^+$ DCs and CD14$^+$CD11b$^+$ monocytes/macrophages, but not CD303$^+$ pDCs. Unexpectedly, by using a number of assays, we could not detect any IL-12p70 production either by tonsil slan/M-DC8$^+$ DCs or by all other tonsil cell populations under investigations, even after their stimulation with LPS or R848 in the presence of IFNγ. The reasons for such inability to produce IL-12p70 are still unclear and need to be clarified at molecular level. We hypothesize that a general desensitization towards bacterial stimuli [35] might occur in inflamed tonsils continuously exposed to bacteria and their products. This might also explain the concomitant poor responsiveness to LPS/R848 plus IFNγ by *ex vivo* tonsil slan/M-DC8$^+$ DCs in terms of TNFα production. Moreover, we explored the possibility that IL-10, readily detectable in our tonsil-conditioned medium, might play a role in determining an inability to produce IL-12p70 by tonsil slan/M-DC8$^+$ DCs. However, addition of anti-IL-10 monoclonal antibodies did not restore the capacity to produce IL-12p70 by TDCM-conditioned slan/M-DC8$^+$ cells, suggesting that other downregulatory mechanisms are likely involved.

Another novel finding of this study is the identification of a remarkable plasticity exhibited by blood slan/M-DC8$^+$ cells. In fact, we show that blood slan/M-DC8$^+$ cells exquisitely acquire all characteristics/features of *ex vivo* isolated tonsil slan/M-DC8$^+$ DCs, including morphology, marker expression and functions when conditioned by TDCM for 5 days. In such regard, we found that TDCMs contain discrete amounts of GM-CSF, but not IL-4, which in concert with other factors might drive slan/M-DC8$^+$ cell differentiation within the tonsil microenvironment. By contrast, we found that blood slan/M-DC8$^+$ cells display a more macrophage-like phenotype when incubated with IL-34. To our knowledge, these are the first data describing effects of IL-34 on circulating slan/M-DC8$^+$ cells, which also express the highest levels of CD115/CSF1R among blood leukocytes. CD115 mRNA is highly restricted to the macrophage lineage [36], whose circulating precursors, at least in mice, are the so-called "patrolling" monocytes [37], known to correspond to the "non-classical" CD14^{dim}CD16$^+$ monocytes in humans [13]. Taken together, all these observations are consistent with the hypothesis of blood slan/M-DC8$^+$ cells as a subset of "non-classical" monocytes [12-13] prone to fully differentiate into a more "DC-like" or "macrophage-like" cells depending on the microenvironment of the colonized tissue. In line with this notion, our data indeed show how blood slan/M-DC8$^+$ cells differentiate into DCs upon migration into tonsils, as also suggested by de Baey *et al.* [17], who firstly described a M-DC8$^+$ cell population in mucosa-associated lymphoid tissues. More broadly, the vision of slan/M-DC8$^+$ cells as a yet not fully differentiated subpopulation of blood CD16$^+$ monocytes, whose fate is driven by local stimuli, can reconcile the debate in the literature on the identity of these cells. In fact, although blood slan/M-DC8$^+$ cells overlap with CD14^{dim}CD16$^+$ non-classical monocytes, tonsil slan/M-DC8$^+$ DCs look and behave differently from their circulating counterpart, displaying bona fide DC functional properties. Despite the definition of slan/M-DC8$^+$ cell ontogeny is beyond the scope of this paper, we speculate for a role of blood slan/M-DC8$^+$ cells as a potential reservoir of tonsil DCs and spotlight their plasticity and commitment under specific tissue microenvironment. Future studies should be aimed at establishing whether such slan/M-DC8$^+$ cell plasticity could be also exploited for therapeutic manipulation of T cell functions in different disease settings.

MATERIALS AND METHODS

Cell isolation and culture

PBMCs were isolated from buffy coats of healthy donors by density centrifugation (Ficoll-Paque; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) under endotoxin-free conditions. Then, slan/M-DC8$^+$ cells, CD1c$^+$ DCs and CD14$^+$ monocytes were purified using specific isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), to more than 90 % purity, while CD4$^+$ T lymphocytes cells were isolated (> 95 % purity) by the EasySep Human CD4 T Cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) [11]. Tonsil samples were obtained from children affected by recurrent, chronic tonsillitis, thus undergoing surgery via cold steel dissection. Tonsils were immediately processed, minced into small fragments, treated for 15 min at 37° with 0.2 mg ml^{-1} Liberase Blendzyme 2 (Roche, Basel, Switzerland), and then processed by gentleMACS

dissociator (Miltenyi Biotec) [11]. Tonsil cell suspensions were washed, filtered through a 40 µm cell strainer and ultimately depleted of T and B lymphocytes by CD3 and CD19 MicroBeads (Miltenyi Biotec), to enrich the DCs. Thereafter, tonsil slan/M-DC8$^+$ DCs, CD1c$^+$ DCs, CD141$^+$ DCs, CD14$^+$CD11b$^+$ monocytes/macrophages and CD303$^+$ pDCs were isolated to more than 90 % purity, by fluorescence activated cell sorting (FACS), using a FACSAria II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). After purification, cells were suspended in standard medium [RPMI 1640 medium supplemented with 10 % low-endotoxin fetal bovine serum (FBS, < 0.5 endotoxin U ml^{-1}, Sigma-Aldrich, St. Louis, MO)] and cultured for 24 h with 100 U ml^{-1} IFNγ (R&D Systems, Minneapolis, MN) in combination with either 5 µM R848 (InvivoGen, San Diego, CA) or 100 ng ml^{-1} ultrapure LPS (from *E. coli*, 0111:B4 strain, Alexis Biochemicals, San Diego, CA). Alternatively, cells were cultured for 5 days in either tonsil-derived conditioned medium, 50 ng ml^{-1} GM-CSF plus 20 ng ml^{-1} IL-4 (both from Miltenyi Biotec), or 100 ng ml^{-1} IL-34 (R&D system). For morphological analysis, cells were subjected to cytospin and stained by the May-Grunwald/Giemsa procedure. Pictures were taken using a Leica DFC 300FX Digital Color Camera on a Leica DM 6000 B microscope. All experimental procedures were approved by the institutional review boards of the University of Verona and Spedali Civili of Brescia. Retrospective analysis of archival material (see below) was conducted in compliance with the Declaration of Helsinki and with policies approved by the Ethics Board of Spedali Civili di Brescia. Human samples were obtained following informed written consent.

Immunohistochemistry

Tissue blocks containing formalin-fixed paraffin-embedded (FFPE) tonsils were retrieved from the tissue bank of the Department of Pathology (Spedali Civili di Brescia, Brescia, Italy). Four-micron thick tissue sections were used for immunohistochemical staining. slan/M-DC8$^+$ cells were specifically identified by using primary antibodies towards the 6-sulfo LacNAc residue (slan/M-DC8) on PSGL-1, namely clone DD1, as previously reported [10]. Other antigens were identified using antibodies listed in Supplementary Table S1. The primary immune reaction was revealed using Novolink Polymer (Leica Microsystems, Wetzlar, Germany) followed by 3, 3'-diaminobenzidine (DAB). For double immunohistochemistry, after completing the first immune reaction, the second one was visualized using Mach 4 MR-Alkaline Phosphatase (AP) (Biocare Medical), followed by Ferangi Blue (Biocare Medical, Concord, CA) as chromogen. For triple immunohistochemistry, after completing the second immune reaction, sections were incubated with primary antibodies to Ki-67 and revealed using a biotinylated system followed by streptavidinconjugated with AP (Dako, Glostrup, Denmark) with New Fucsin as chromogen.

Generation of tonsil derived conditioned medium (TDCM) and TDCM-conditioned cells

TDCM was generated by culturing tonsil cell suspension (10*10^6 ml^{-1}, n = 8) in RPMI plus 10 % FBS for 24 h. Cell-free supernatants were then collected and stored at - 20° C. Each TDCM was diluted 1:5 in RPMI plus 10 % FBS immediately before its addition to blood slan/M-DC8$^+$ cells, CD1c$^+$ DCs or CD14$^+$ monocytes for subsequent incubation. After 5 d, cells were harvested, extensively washed and used for different functional assays. In selected experiments, anti-IL-10 mAbs, or their IgG2a isotype controls (10 µg ml^{-1}, both from R&D system), were added to slan/M-DC8$^+$ cells during the 5 d-incubation with TDCM, as well as during the subsequent 24 h-activation with IFNγ plus LPS.

Flow cytometry analysis

For phenotypic studies, typically 2.5x10^5 PBMCs, 5x10^5 cells from tonsil cell suspensions or 10^4 *in vitro* stimulated slan/M-DC8$^+$ cells were initially incubated for 10 min in 50 µl Phosphate Buffer Solution (PBS) containing 5 % human serum (to prevent nonspecific binding), and then stained for 15 min at room T using the monoclonal antibodies listed in Supplementary Table S2. Sample fluorescence was measured by an eight-color MACSQuant Analyzer (Miltenyi Biotec), while data analysis was performed by FlowJo software Version 8.8.7 (Tree Star Inc., Stanford, CA) [11]. Cell viability was analyzed using Vybrant® DyeCycle™ Violet (Life Technologies, Carlsbad, CA) [11]. Phenotypic analysis under the various experimental conditions was performed on live cells, identified as Vybrant-negative cells (in the case of TDCM-conditioned/stimulated slan/M-DC8$^+$ cells) or PI-negative cells (in the case of tonsil cell suspensions) [11]. The mean fluorescence intensity (MFI) relative to each molecule was obtained by subtracting either the MFI of the correspondent isotype control, or cell autofluorescence (fmo).

T cell proliferation assays

For allogeneic assays, 1.25-5x10^3 slan/M-DC8$^+$ DCs, CD1c$^+$ DCs, CD141$^+$ DCs, CD14$^+$CD11b$^+$ monocytes/macrophages and CD303$^+$ pDCs, sorted from tonsils, were co-cultured with 5x10^4 CFSE-labeled allogeneic CD4$^+$ T lymphocytes in U-bottom 96-well plates [11]. For autologous assays, 5x10^3 freshly isolated or 5-d TDCM-conditioned slan/M-DC8$^+$ cells, CD1c$^+$ DCs and CD14$^+$ monocytes were co-cultured with 5x10^4 CFSE-labeled

autologous CD4+ T lymphocytes in U-bottomed 96-well plates, in the absence or presence of 5 µg ml^{-1} tetanus toxoid (TT) [11]. For both allogeneic and autologous assays, T-cell proliferation was assessed after 7 days by measuring CFSE dilution by flow cytometry [11].

Analysis of cytokine production

Total cell suspensions from tonsils were analyzed for TNFα and IL-12p70 production by specific cytokine secretion assays (Miltenyi Biotec) [11]. Briefly, $5x10^5$ tonsil cells were incubated with 100 U ml^{-1} IFNγ in combination with either 100 ng ml^{-1} ultrapure LPS or 5 µM R848 in standard medium at 37°C either for 4 h, to optimally detect TNFα secretion, or for 12 h, after a 6 h pre-incubation in standard medium, to optimally detect IL-12p70 secretion. Percentages of cytokine secreting cells were then identified as cytokine-positive cells among total slan/M-DC8+ DCs, CD1c+ DCs, CD14+CD11b+ monocytes/macrophages and CD303+ pDCs, gated as shown in detail in Supplementary Figure S1. TNFα and IL-12p70 levels present in cell-free supernatants harvested from either blood or TDCM-conditioned slan/M-DC8+ cells, and stimulated as detailed in legend to Figure 6, were measured by specific ELISA kits from eBioScience (San Diego, CA; sensitivity: 4 pg ml^{-1}). The levels of IL-10, GM-CSF and IL-4 in TDCMs were measured by ELISA kits, purchased from eBioScience, BioLegend (San Diego, CA) and Mabtech (Cincinnati, OH), respectively. Detection limits of these ELISA were: 2 pg ml^{-1} for IL-10, 3 pg ml^{-1} for GM-CSF and IL-4

Statistical analysis

Data are expressed as means ± SEM of the number of experiments indicated in each Figure legend. Statistical analysis, including one-way or two-way analysis of variance followed by Bonferroni's post hoc test, was performed by Prism Version 5.0 software (GraphPad Software, Inc., La Jolla, CA).

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CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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4.1.2 SUPPLEMENTAL MATERIAL FOR:

slan/M-DC8^{+} cell constitute a distinct subset of dendritic cells in human tonsils

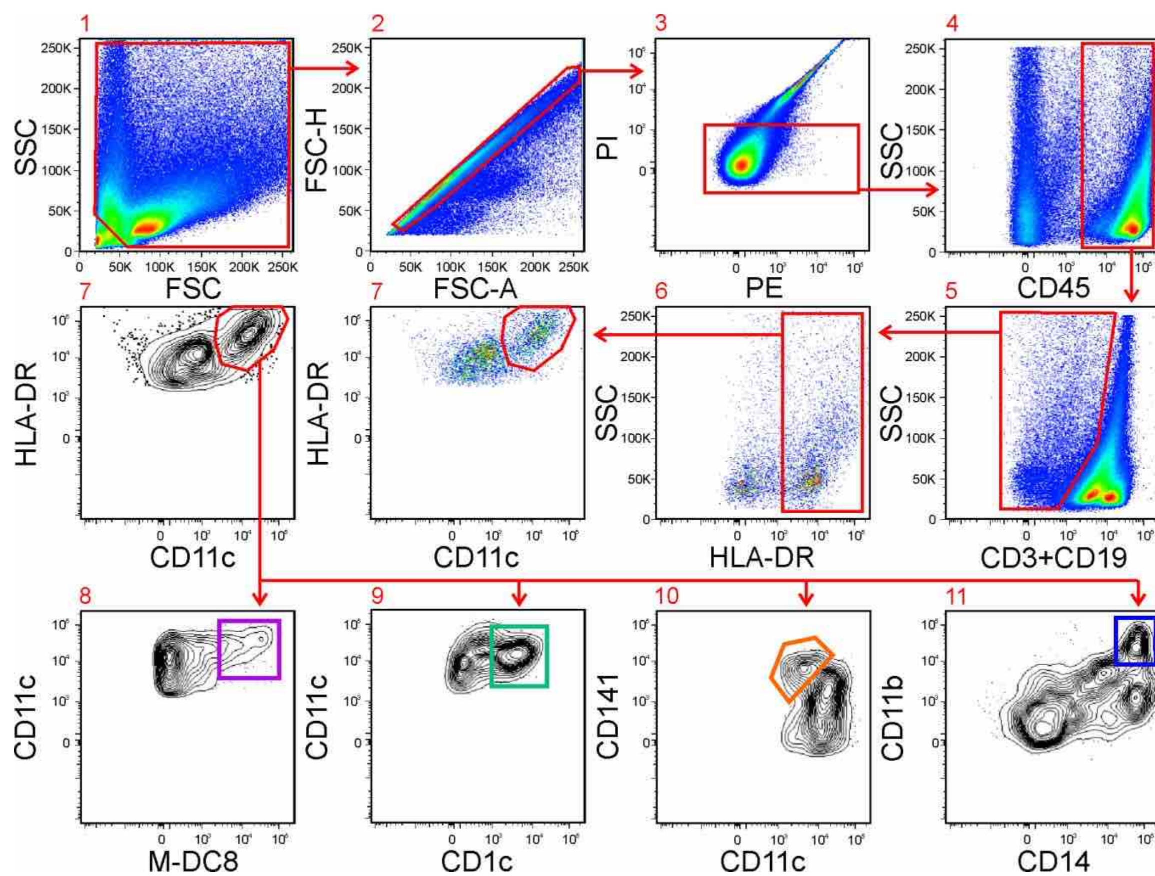
slan/M-DC8$^+$ cells constitute a distinct subset of dendritic cells in human tonsil

Supplementary Table S1. List of the antibodies used for immunohistochemistry studies.

Reagent	Clone	Dilution	Isotype	Source
BCL6	IG191E/A8	1:300	mIgG1	kindly provided by G. Roncador (Centro Nacional de Investigaciones Oncológicas Madrid, Spain)
CD1a	010	1:50	mIgG1	Dako
CD3	SP7	1:100	rabbit	Thermo Scientific, Waltham, MA
CD4	4B12	1:40	mIgG1	Thermo Scientific
CD8	C8/144B	1:30	mIgG1	Dako
CD11b		1:300	rabbit polyclonal	Sigma-Aldrich
CD14	7	1:50	mIgG2a	Novocastra Laboratories, Newcastle upon Tyne, United Kingdom
CD66b	G10F5	1:200	mIgM	BioLegend, San Diego, CA
CD83	1H4b	1:150	mIgG1	Novocastra Laboratories
DD1	DD1	1:60	mIgM	kindly provided by Knut Schäkel (University Hospital Heidelberg, Heidelberg, Germany)
Keratin (wide spectrum-CKP)	MNF116	1:100	mIgG1	Dako
Ki-67	MM1	1:100	mIgG1	Novocastra Laboratories

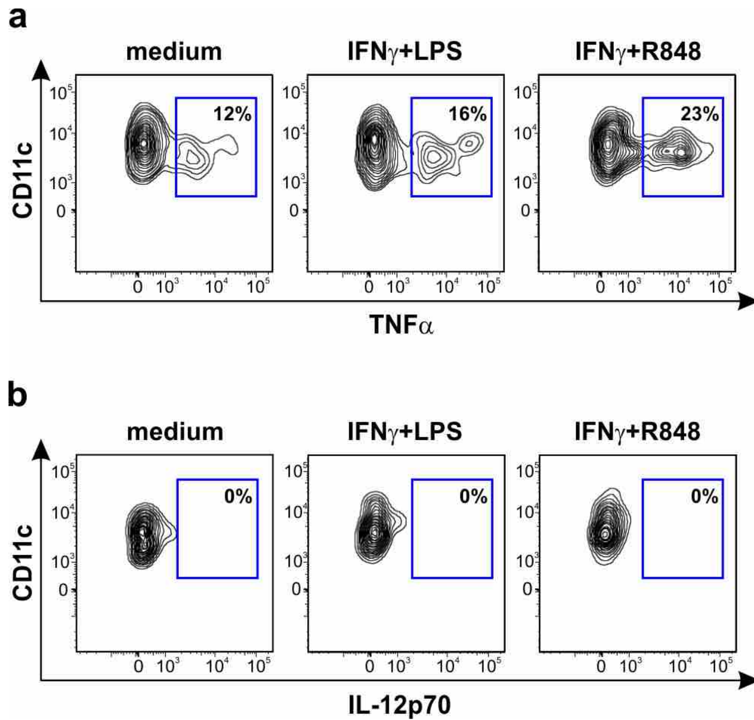
Supplementary Table S2. List of the antibodies used for flow cytometry.

Antibody	Clone	Isotype	Source
AlexaFluor488 anti-human CD1c	L161	mIgG1	BioLegend
AlexaFluor647 anti-human CX3CR1	2A9-1	rat IgG2b	BioLegend
AlexaFluor647 rat IgG2b	RTK4530		BioLegend
APC anti-human CD11b	ICRF44	mIgG1	BioLegend
APC anti-human CD11c	MJ4-27G12	mIgG2b	Miltenyi Biotec
APC anti-human CD14	TUK4	mIgG2a	Miltenyi Biotec
APC anti-human CD141 (BDCA-3)	AD5-14H12	mIgG1	Miltenyi Biotec
APC-Cy7 anti-human HLA-DR	L243	mIgG2a	BioLegend
Brilliant Violet 510 anti-human CD45	HI30	mIgG1	BioLegend
FITC anti-human CD14	TÜK4	mIgG2a	Miltenyi Biotec
FITC anti-human CD141	AD5-14H12	mIgG1	Miltenyi Biotec
FITC anti-human CD303	AC144	mIgG1	Miltenyi Biotec
FITC anti-human Slan (M-DC8)	DD1	mIgM	Miltenyi Biotec
PE anti-human CD1c (BDCA-1)	AD5-8E7	mIgG2a	Miltenyi Biotec
PE anti-human CD11b	ICRF44	mIgG1	BioLegend
PE anti-human CD115	9-4D2-1E4	rat IgG1	BioLegend
PE anti-human CD14	TUK4	mIgG2a	Miltenyi Biotec
PE anti-human CD16	3G8	mIgG1	BioLegend
PE anti-human CD163	GHI/61	mIgG1	BioLegend
PE anti-human CD40	HB14	mIgG1	BioLegend
PE anti-human CD80	2D10	mIgG1	BioLegend
PE anti-human CD83	HB15	mIgG1	Miltenyi Biotec
PE anti-human CD86	IT2.2	mIgG2b	BioLegend
PE anti-human FcεRI	CRA1	mIgG2b	Miltenyi Biotec
PE anti-human CD206	15-2	mIgG1	BioLegend
PE anti-human CD209 (DC-SIGN)	9E9A8	mIgG2a	BioLegend
PE mouse IgG1	MOPC-21	mIgG1	BioLegend
PE-Cy7 anti-human CD19	HIB19	mIgG1	BioLegend
PE-Cy7 anti-human CD3	UCHT1	mIgG1	BioLegend
PerCP-Cy5.5 anti-human CD16	3G8	mIgG1	BioLegend
Vioblue anti-human CD11c	MJ4-27G12	mIgG2b	Miltenyi Biotec



Supplementary Figure S1. Gating strategy to distinctively identify slan/M-DC8$^+$ DCs, CD1c$^+$ DCs, CD141$^+$ DCs and CD14$^+$CD11b$^+$ monocytes/macrophages in human tonsils.

Single cell suspensions from tonsils were processed for flow cytometry analysis to identify slan/M-DC8$^+$ DCs, CD1c$^+$ DCs, CD141$^+$ DCs and CD14$^+$CD11b$^+$ monocytes/macrophages. Steps 1-4 were sequentially used to exclude cell debris (1), doublets (2), dead cells (3), and, ultimately, to gate CD45$^+$ leukocytes (4). Subsequently, in steps 5-7, analysis was performed on CD3/CD19-negative cells (5). Within the latter cells, HLA-DR-positive (6) and subsequently HLA-DR$^+$CD11c$^+$ cell populations (7) were gated. The latter HLA-DR$^+$CD11c$^+$ population includes, in fact, all myeloid DCs, macrophages and monocytes. Steps 8-11 show the specific combination of markers used to gate each myeloid population type: slan/M-DC8$^+$ DCs (purple gate, 8), CD1c$^+$ DCs (green gate, 9), CD141$^+$ DCs (orange gate, 10) and CD14$^+$/CD11b$^+$ monocytes/macrophages (blue gate, 11).



Supplementary Figure S2. TNFα and IL-12p70 secretion by tonsil slan/M-DC8^{+} DCs.

Tonsil cell suspensions were incubated with or without 100 U ml^{-1} IFNγ plus either 100 ng ml^{-1} LPS or 5 μM R848, either for 4 h (to detect TNFα secretion), or for 12 h, after a 6 h pre-incubation (to detect IL-12p70 secretion). Contour plots display a representative experiment illustrating the percentage of TNFα- (a) or IL-12p70- (b) secreting tonsil slan/M-DC8^{+} DCs.