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# Etude du rôle des Interférons de type III dans le microenvironnement tumoral

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# THESE de DOCTORAT DE L'UNIVERSITE CLAUDE BERNARD LYON 1

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## **Etude du rôle des Interférons de type III dans le microenvironnement tumoral**

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## **RESUME EN FRANCAIS :**

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Pour se protéger des infections bactériennes ou virales, mais aussi pour empêcher le développement tumoral, l'organisme met en place une réponse immunitaire. Le système immunitaire est composé de molécules et de cellules impliquées dans la réponse immunitaire innée et adaptative. Les premiers acteurs engagés dans la défense de l'hôte sont les cellules NK, les monocytes et macrophages et les granulocytes qui comprennent les neutrophiles. La réponse immunitaire innée permet de contrôler rapidement tous pathogènes ou cellules anormales de façon non spécifique. Au contraire, la réponse immunitaire adaptative est quant à elle spécifique et arrive plus tardivement après la détection d'un corps étranger. Cependant, pour une réponse immunitaire appropriée un lien doit être fait entre les cellules de l'immunité innée et adaptative. Ce lien est fait par les cellules présentatrices d'antigène (APC) dont font partie les cellules dendritiques (DCs).

Il existe différentes populations de DCs. Les cinq grandes familles de DCs sont : les cellules dendritiques conventionnelles de type 1 (cDC1) et de type 2 (cDC2), les cellules de Langerhans (LC), les DCs dérivées de monocytes (monoDC) et enfin, les cellules dendritiques plasmacytoides (pDCs). Les pDCs possèdent trois propriétés immunostimulatrices : la présentation d'antigène, leur cytotoxicité directe, et leur production d'interférons type I (IFN-I). Les IFN-I sont connus pour leurs propriétés antivirales, mais aussi pour leur capacité à augmenter les actions des autres cellules immunitaires. Les IFN-I sont donc d'une importance capitale dans la réponse antitumorale. Cependant les pDCs sont souvent inhibées par différents éléments du microenvironnement tumoral (TME), tel que les TGF- $\beta$ , et ne produisent plus d'IFN-I. Certaines nouvelles immunothérapies visent à activer les pDCs pour permettre la restauration de leurs propriétés immunostimulatrices tel que leur présentation d'antigène et leur production d'IFN-I. Bien que ces stratégies induisent de bonnes réponses immunitaires, la toxicité de ces traitements sont encore trop élevées pour qu'ils puissent être validés. Il est donc important de développer de nouvelles stratégies d'immunothérapies visant à activer les pDCs associées aux tumeurs (TA-pDCs) pour augmenter leur production d'IFN-I, tout en diminuant les effets toxiques du traitement.

Notre équipe a démontré que la présence d'IFN de type III (IFN-III) dans les tumeurs de sein était associée avec un meilleur pronostic. Les IFN-III est la famille de cytokines la plus récemment découverte et comprend IFN- $\lambda$ 1, IFN- $\lambda$ 2, et IFN- $\lambda$ 3, aussi connue en tant qu'IL-29, IL-28B, IL-28A. IFN-III interagissent avec le récepteur IFN $\lambda$ R, un hétérodimère composé de la chaîne IFN $\lambda$ R1 et IL-10R $\beta$ . Mon projet de thèse avait donc pour objectif de (1) déterminer

quelles cellules immunitaires répondent aux IFN-III, (2) déterminer l'impact que les IFN-III ont sur les cellules qui répondent en termes de fonctionnalité, (3) quel pourrait être l'effet d'un traitement d'immunothérapie utilisant les IFN-III sur les cellules associées à la réponse immunitaire antitumorale.

Au cours, de ma thèse nous avons pu déterminer que les pDCs sont les cellules qui répondent le plus fortement aux IFN-III. Les IFN-III augmentent la survie des pDCs, mais augmente aussi la présence de marqueurs d'activation. Nous avons pu déterminer que les IFN-III augmente l'expression de TLR7 par les pDCs, les rendant plus sensible de faible dose de TLR7 agoniste. Après stimulation avec des IFN-III et une activation avec un TLR7 agoniste, les pDCs ont une très fortement augmentation de la sécrétion d'IFN-I. Enfin, nous avons voulu savoir si cette forte sécrétion d'IFN-I était maintenue en présence de TGF- $\beta$ , cytokine inhibitrice de pDCs. Il s'avère que la présence d'IFN-III dans le milieu de culture de pDCs empêche l'inhibition du TGF- $\beta$ . Notre équipe avait aussi démontré que les cDC1 étaient les seules cellules productrices d'IFN-III dans le TME. En analysant la localisation des pDCs et des cDC1 sur des coupes de tumeur de sein, nous avons pu constater que les TA-pDCs sont situées à proximité des TA-cDC1. Les TA-pDCs pourrait donc bénéficier des IFN-III sécrétée par les cDC1 pour augmenter leurs capacités à sécréter des IFN-I.

En conclusion, nos résultats (1) mettent en avant les mécanismes induit par les IFN-III chez les pDCs pour augmenter leur production d'IFN-I et, (2) renforce la possibilité d'utiliser les IFN-III comme immunothérapie, car ils pourraient permettre de restaurer la production d'IFN-I par les TA-pDCs avec l'utilisation de dose plus faible de TLR7 agoniste.

**Mot clés : pDCs, IFN-I, IFN-III, TLR7, TME, Immunotherapie**

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## **RESUME EN ANGLAIS / ABSTRACT :**

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### **Exploring the role of type III interferons on the tumor microenvironment**

To protect itself from bacterial or viral infections, but also to prevent tumor development, the organism sets up immune responses. The immune system is composed of molecules and cells involved in the innate and adaptive immune system. The first actors involved in host defense are NK cells, monocytes, macrophages and granulocytes which include neutrophils. The innate immune response quickly controls the proliferation of any pathogens or abnormal cells in a non-specific way. On the contrary, the adaptive immune response is specific and comes later after the detection of an abnormality. However, for an appropriate immune response a link must be made between innate and adaptive immune system. This link is made by antigen-presenting cells (APC) and dendritic cells (DCs) belong to this category.

There are different DC subsets. The five main subsets are: type 1 and type 2 conventional dendritic cells (cDC1 and cDC2), Langerhans cells (LC), monocyte-derived DCs (monoDC) and finally, plasmacytoid dendritic cells (pDCs). pDCs have three immunostimulatory properties: antigen presentation, direct cytotoxicity, and type I interferons (IFN-I) production. IFN-I are known for their antiviral properties, but also for their ability to increase the actions of other immune cells. IFN-I are therefore of major importance in the antitumor response. However, pDCs are often inhibited by different elements of the tumor microenvironment (TME), such as TGF- $\beta$ , and no longer produce IFN-I. new immunotherapies aim to activate pDCs to allow the restoration of their immunostimulatory properties such as their antigen presentation and their production of IFN-I. Although these strategies induce good immune responses, the toxicity of these treatments are still too high to be validated. It is therefore important to develop new immunotherapy strategies to activate tumor-associated pDCs (TA-pDCs) to increase their production of IFN-I, while reducing the toxic effects of the treatment.

Our team has demonstrated that the presence of type III IFN (IFN-III) in breast tumors is associated with a good prognosis. IFN-III is the most recently discovered family of cytokines and includes IFN- $\lambda$ 1, IFN- $\lambda$ 2, and IFN- $\lambda$ 3, also known as IL-29, IL-28B, IL-28A. IFN-III interact with the IFN $\lambda$ R receptor, a heterodimer composed of the IFN $\lambda$ R1 and IL-10R $\beta$  chain. Therefore, my PhD project aimed to (1) determine which immune cells respond to IFN-III, (2) determine the impact that IFN-III has on the cells that respond in terms of functionality, what

could be the effect of an immunotherapy treatment using IFN-III on the cells associated with the antitumor immune response.

During my PhD we were able to determine that pDCs are the cells that respond best to IFN-III. IFN-III increases pDC survival, but also increases activation markers on pDCs. We were able to determine that IFN-III increases the expression of TLR7 by pDCs, making them more sensitive to low dose TLR7 agonist. After stimulation with IFN-III and activation with a TLR7 agonist, pDCs have a very strong increase in IFN-I secretion. Finally, we wanted to know if this strong secretion of IFN-I was maintained in the presence of TGF- $\beta$ , a cytokine that inhibits pDCs. Adding IFN-III pDCs culture prevents the inhibition of TGF- $\beta$ . Our team had also demonstrated that cDC1 were the only IFN-III producing cells in the TME. By analyzing the localization of pDCs and cDC1 on breast tumor sections, we were able to observe that TA-pDCs are located close to TA-cDC1. TA-pDCs could therefore benefit from IFN-III secreted by cDC1 to increase their capacity to secrete IFN-I.

In conclusion, our results (1) highlight the mechanisms induced by IFN-III in pDCs to increase their production of IFN-I and, (2) reinforce the possibility of using IFN-III as immunotherapy, because they could make it possible to restore the production of IFN-I by the TA-pDCs with the use of a lower dose of TLR7 agonist.

**Key words : pDCs, IFN-I, IFN-III, TLR7, TME, Immunotherapy**

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## **ABBREVIATIONS**

### **A**

**APC** : Antigen Presenting Cells  
**ADAR** : RNA-specific adenosine deaminase  
**APRIL** : A proliferation-inducing ligand

### **B**

**BAFF** : B cell activating factor  
**BCC** : basal cell carcinoma  
**BDCAs** : Blood DC Antigens  
**BLIMP-1** : B-Lymphocyte-induced maturation protein 1

### **C**

**CBF $\beta$**  : Core-binding factor subunit  $\beta$   
**cDCs** : conventional Dendritic Cells  
**cDC1** : Type 1 conventional Dendritic Cells  
**cDC2** : Type 2 conventional Dendritic Cells  
**CDP** : Common DC Progenitor  
**CLP** : Common Lymphoid Progenitor  
**CRS**: Cytokine release syndrome  
**cSCC** : squamous Cell Skin Cancer

### **D**

**DAMP** : Damage Associated Molecular Pattern  
**DCs** : Dendritic Cells  
**DEG** : Differentially Expressed Genes

### **E**

**EMA** : European Medical agency

### **F**

**FLT3L** : FMS-like tyrosine kinase 3 ligand  
**FDA** : Food and Drug Administration

### **H**

**HCS** : hematopoietic stem cell  
**HIV** : Human immunodeficiency virus  
**HSPC** : hematopoietic stem and progenitor  
**HNSCC** : head and neck squamous cell carcinoma  
**HPV** : Human papillomavirus

### **I**

**ICI** : Immune Checkpoint Inhibitor  
**IDO**: Indoleamine 2.3 Dioxygenase  
**IFN-I** : Type I Interferon  
**IFN-II** : Type II Interferon  
**IFN-III** : Type III Interferon  
**IMQ** : Imiquimod  
**IRF** : interferon regulatory factor  
**ISG** : Immune Stimulated Genes  
**ISRE** : interferon stimulated response element

### **L**

**LC** : Langherans Cells

### **M**

**MAPK** : Mitogen-associated protein kinase  
**M $\emptyset$**  : Macrophages  
**MHC-I** : Major Histocompatibility Complex class I  
**MHC-II** : Major Histocompatibility Complex class II  
**moDCs** : monocyte-derived DCs

### **N**

**NSCLC** : Non-Small Cell Lung Cancer

## O

**OPSCC** : oropharyngeal squamous cell carcinoma

## P

**PAMP** : Pathogen Associated Molecular Pattern

**PBMCs** : peripheral blood mononuclear cells

## R

**RCC**: renal cell carcinoma

**RNA seq** : RNA sequencing

## S

**sc-RNA seq** : single-cell RNA sequencing

**SLOs** : secondary lymphoid organs

**siRNA** : using small interfering RNA

**SOCS** : Suppressor Of Cytokines Signaling

**SNP** : single nucleotide polymorphism

## T

**TA** : Tumor-Associated

**TAA** : Tumor associated Antigen

**TAM** : Tumor-associated Macrophages

**Tfh** : T follicular helper cell

**Th** : T helper

**TLS** : tertiary lymphoid structures

**TRAIL** : Tumor-necrosis-factor related apoptosis inducing ligand

**Tregs** : regulatory T cells

## V

**VSV** : vesicular stomatitis virus

**PD-1** : programmed cell death protein 1

**pDCs** : plasmacytoid Dendritic Cells

**PI3K** : phosphoinositide 3 Kinase

**Pin-1** : peptidyl-propyl cis-trans isomerase NIMA-interacting

**PKR** : double-stranded RNA-activated protein kinase

**PRRs** : Pattern Recognition Receptors

# INTRODUCTION



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## **CHAPTER I**

### *Phenotypes and functions of human Dendritic Cell subsets in the Tumor microenvironment, with a focus on tumor-associated plasmacytoid dendritic cells*

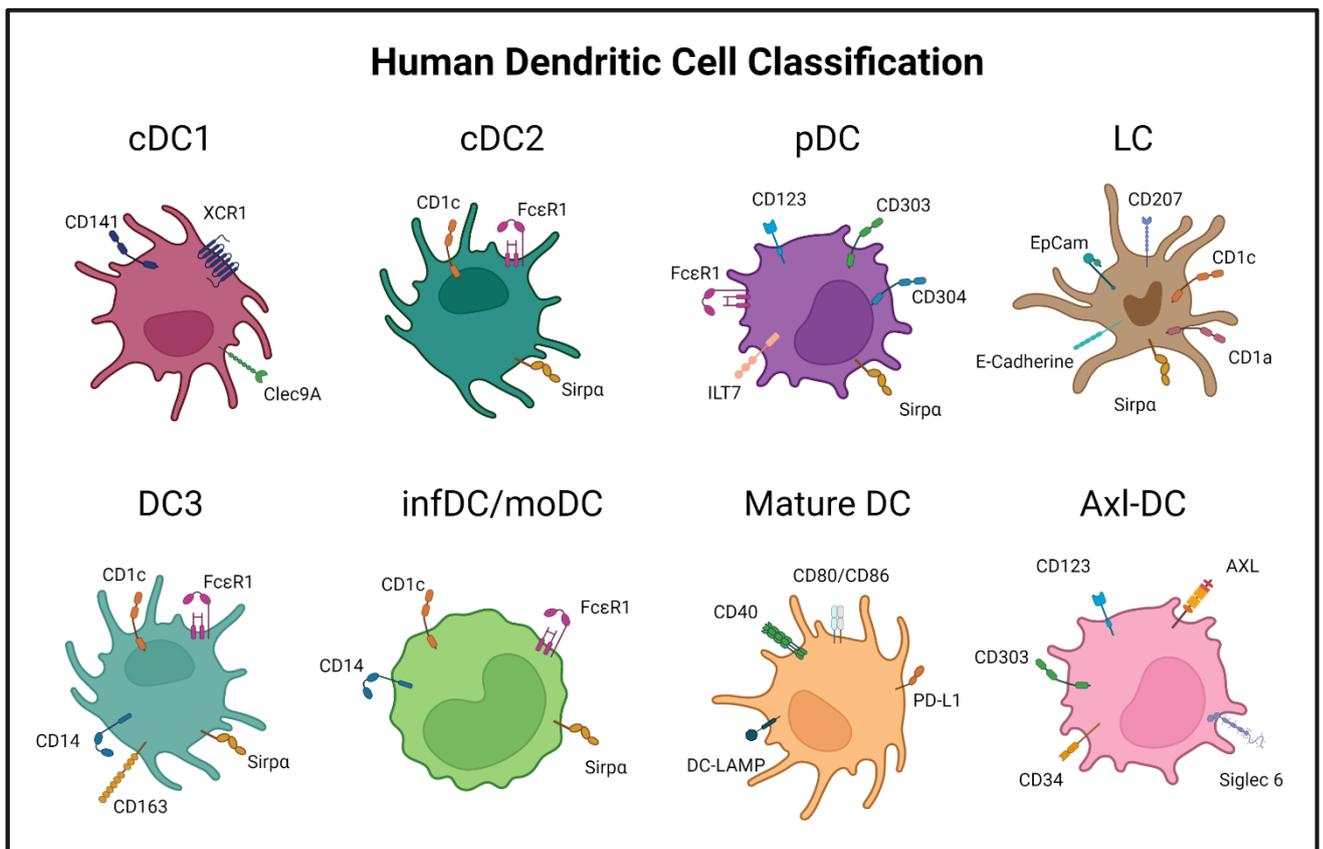
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#### **I. Identification of human DC subsets**

Dendritic cells (DCs) were first identified in humans in the early 80's by R. Steinman & G. Kaplan's team (Van Voorhis et al. 1982). Their high expression of pattern recognition receptors (PRRs) (Akira and Takeda 2004) and their secretion of a wide range of cytokines (Piqueras et al. 2006) give them a central role at the interface between innate and adaptive immune responses (Paul 2011). DCs are also the most efficient antigen-presenting cells (APC). Due to this interesting ability, DCs were intensively investigated in the past fifty years using flow cytometry, *in situ* hybridization, immunofluorescence, and RNA sequencing methods (at bulk or single-cell levels). These approaches helped to understand how DCs orchestrate adaptive immune responses against infectious diseases and tumors (Banchereau and Steinman 1998). These methods also helped us to appreciate the important diversity of DC subsets from which a universal consensus has emerged about their classification in both humans and mice. DCs are indeed commonly separated into plasmacytoid dendritic cells (pDCs), and conventional dendritic cells (cDCs), the latter including type 1 cDCs (cDC1) and type 2 cDCs (cDC2). Langerhans cells (LC) are often associated with DCs but they are actually a population of tissue-resident macrophages (Doebel, Voisin, and Nagao 2017). It has been described that cDCs as well as pDCs differentiated from the common DC progenitor (CDP) even though there is evidence showing that pDCs could also derive from the common lymphoid progenitor (CLP) (Dress et al. 2019; Musumeci et al. 2019). A new DC subset was recently described in human blood by *Villani et al.*, the Axl-DC subsets that may be related to pDCs because of its lineage. Coming from another differentiation pathway (Geissmann et al. 2010), we should also consider the family of monocyte-derived DCs (moDCs) which are generated from monocytes under

inflammatory conditions (León, López-Bravo, and Ardavin 2007). Recently a new population of DC was identified as DC3 (Dutertre et al. 2019). They are closely related to cDC2 but can be identified as: CD5- CD163+ CD14+ DCs. The high heterogeneity among the DC family makes the concomitant analysis of all DC subsets difficult, especially when DCs are very rare within complex tissues like tumors. Here we discuss human DC heterogeneity in human blood and tumors based on their phenotypes, functions, and localization.

**Figure 1 – Human Dendritic Cell Classification**



Human dendritic cells can be divided into 8 major subsets: cDC1, cDC2, pDC, LC, Mature DC, moDC, Axl-DC, and DC3. Each subset expresses different surface markers helping the analysis of their function in the TME thanks to different technologies. The different subsets can share similar surface markers. Notably, cDC2 and LC have in common the expression of BDCA1/CD1c, and Sirpa/CD172a. However, LC can be distinguished thanks to the expression of Langerin/CD207, EpCam, E-cadherin, and CD1a. Similarly, pDCs and Axl-DCs share the expression of IL-3R/CD123 and BDCA2/CD303 but exclusively express BDCA4/CD304, Sirpa/CD172a, ILT7, FcεR1 for pDCs, and AXL, Siglec-6, for Axl-DC. cDC1 can be easily identified through their specific expression of Clec9A and XCR1, as well as their higher expression BDCA3/CD141 compared to other DC subsets. DC3 can be differentiated from cDC2 with expression of CD14 and CD163. However, it would be complicated to differentiate them from moDCs if it was not for their expression of CD163. Finally, mature DCs can be differentiated from other DC subsets with their unique expression of DC-LAMP.

## **A. Conventional dendritic cells**

Conventional DCs (cDCs), also known as myeloid DCs (mDCs), are found by flow cytometry in the fraction of Lineage-negative (CD3, CD14, CD20, CD15, CD56 negative) cells expressing CD11c as well as high levels of major histocompatibility complex (MHC) class II molecules (HLA-DR) at steady-state. In peripheral blood, cDCs only represent a small fraction of immune cells (2%) when compared to neutrophils, T cells (70-85%), NK cells (5-20%), or B cells (5-10%). The discovery of the expression of blood DC antigens (BDCAs) at the surface of DCs enabled the study of DCs by multiparametric flow cytometry. Indeed, whereas cDC1 are negative for BDCA1 and express high levels of BDCA3, cDC2 are BDCA1 positive and BDCA3-/low **[Figure 1]** and they respectively represent only 0.05% and 0.6% of peripheral blood mononuclear cells (PBMCs).

### **1. Type 1 conventional Dendritic Cells (cDC1)**

Antigen cross-presentation is the ability to load antigen-derived peptides after phagocytosis on MHC-I molecules to activate CD8+ T cells. Among all DC subsets, HLA-DR+ CD11c+ CD141<sup>high</sup> cDC1 excel in antigen cross-presentation for several reasons. They specifically express high levels of Clec9a/CD370, a C-type lectin allowing endocytosis of necrotic cells (Sancho et al. 2009; Ahrens et al. 2012; J.-G. Zhang et al. 2012) and are particularly well-equipped for antigen processing (Théry and Amigorena 2001) to upload antigens on their MHC-I. cDC1 also highly express TLR3 which detects viral double-stranded RNA and facilitates cDC1-mediated cross priming of CD8+ T cells against virus-infected cells (Schulz et al. 2005). However, in order to cross present antigens, cDC1 and CD8+ T cells have to co-localize and physically interact. The expression of XCR1 by cDC1 (Haniffa et al. 2012; Hubert et al. 2020) allows their recruitment through the recognition of XCL1 produced by activated NK cells within the tumor microenvironment (TME) (Böttcher et al. 2018) and CD8+ T cells in the draining lymph node. It also appears that human cDC1 express the lectin NECL2/CADM1, allowing cDC1 interaction with CD8+ T cells (Galibert et al. 2005; Arase et al. 2005; Boles et

al. 2005). In addition, single-cell RNA sequencing (scRNA-seq) data was used to infer cell-cell interaction networks with other immune cells. In this context, *Zhang et al.* have shown that, in colon cancer, cDC1s and T cells may specifically interact through the CCR8-CCL4 axis (Lei Zhang et al. 2020). Finally, cDC1 are responsible for the highest production of IL-12p70 in human (Jongbloed et al. 2010; Lionel Franz Poulin et al. 2010) leading to the polarization of naïve CD4+ T cells into T helper (Th) 1 cells, which secrete IFN- $\gamma$  and subsequently enhance CD8+ T cells activation. These phenotypes and cytokines secretion indicate a close interaction between cDC1 and CD8+ T cells supporting the statement that cDC1 are strongly equipped to perform naïve T cell activation.

## **2. Type 2 conventional dendritic cells (cDC2)**

cDC2 are defined as CD11b+ SIRP $\alpha$ + BDCA1+ CD16+ cells (Miller et al. 2012), and are considered potent activators of CD4+ T cells (Théry and Amigorena 2001; Guermonprez et al. 2002). cDC2 express a large variety of PRRs, such as TLR1, 2, 4, 5, 6, and 8 (Hémont et al. 2013), and multiple lectins, CLEC4A/CD367, CLEC7A/CD369, CLEC6A, CLEC10A, and CLEC12A/CD371 (Collin and Bigley 2018). This very broad panel of innate receptors can explain the high abilities of cDC2 to recognize different types of pathogens and to present and cross-present antigens. They can also secrete cytokines, such as TNF- $\alpha$ , IL-1, IL-6, IL-8, IL-10, IL-12p70, IL-23 (Jin et al. 2014; Nizzoli et al. 2013; Sittig et al. 2016), to polarize naïve CD4+ T cells toward Th1, Th2, or Th17 subsets depending on the type of infection or inflammation (Jin et al. 2014; Blasio et al. 2016; Segura, Durand, and Amigorena 2013; Cohn et al. 2013). In contrast to cDC1 which have well-defined functions, this broad range of receptors and cytokines expressed by cDC2 could be a consequence of their high heterogeneity (Alcántara-Hernández et al. 2017). Indeed, it was reported by scRNA-seq analysis, that in many cancer types the distinction between cDC2 and moDCs is often complicated to decipher (N. Kim et al. 2020) and that cDC2 contains many different clusters of cells. Moreover, in inflammatory context, an upregulation of BDCA3, CD11b, CD16, or CD14 expression can occur (Boltjes and van Wijk 2014) leading to their identification as moDC or

cDC1. cDC2 heterogeneity is so complex that different subsets arise. As mentioned before, the new DC3 (Dutertre et al. 2019) are closely related to cDC2. Further investigations need to be conducted to determine if DC3 are a real distinct subset, or a consequence of cDC2 heterogeneity. Finally, cDC2a and cDC2b subpopulation has also been described in humans (Brown et al. 2019) with cDC2a being associated with anti-inflammatory response and cDC2b with pro-inflammatory response. Thus, identifying specific markers of cDC2, and investigating their heterogeneity, would enhance our capacity to study them further.

## **B. Langerhans Cells (LC)**

Similarly to all DCs, LCs express HLA-DR (Klareskog et al. 1977), and display almost the same phenotype as cDC2 with the expression of MHC-I, SIRP $\alpha$ , BDCA1 and CD16 molecules. However, in humans they also express high level of cell-surface C-type lectin receptors such as CD207 (Valladeau et al. 2000) which help to differentiate them from other CD207<sup>-low</sup> cDC2. Combined with their expression of different proteins involved in their interaction with epithelial cells, such as EpCAM and E-cadherine (Hieronymus et al. 2015), LC can easily be differentiated from cDC2. LC have the capacity i) to interact with epithelial cells (Hieronymus et al. 2015), ii) to capture antigens and activate CD4<sup>+</sup> T cells and induce a Th2 polarization in the draining lymph nodes (Klechevsky et al. 2008), iii) to activate CD8<sup>+</sup> T cells through antigen cross-presentation (Klechevsky et al. 2008; Artyomov et al. 2015).

LC are a population of tissue-resident macrophages and are mainly localized in the skin where all these previous functions have been described. Only few studies assess the role of LC in antitumor response. Our team identified LC within tumors, and their infiltration is associated with an overall survival in multiple humans cancers (Hubert et al. 2020). However, a review evaluating the density of LC in nonmelanoma skin cancer is more divided concerning LC infiltration in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (Pogorzelska-Dyrbuś and Szepietowski 2020). Finally, in humans cutaneous squamous cell carcinoma, LC can induce strong Type 1 immunity (Fujita et al. 2012).

### **C. Plasmacytoid Dendritic Cells**

pDCs can be separated from cDCs thanks to their surface expression of BDCA2/CD303 and identified by flow cytometry as HLA-DR<sup>+</sup> CD11c<sup>-</sup> BDCA2<sup>+</sup> CD123<sup>+</sup> BDCA4<sup>+</sup> SIRP $\alpha$ <sup>+</sup> cells. While pDCs are known to be less capable than cDCs to prime T cells, their major function is to secrete high amount of type I interferons (IFN-I) (Marina Cella et al. 1999; Siegal et al. 1999) after TLR7 and TLR9 activation by viral ssRNA and ODN CpG respectively. In 2018, *Alculumbre et al.* distinguished three pDC subpopulations with variable capacities of cytokine secretion and antigen presentation, as well as different levels of expression of activation markers such as CD80 and PD-L1 (Alculumbre et al. 2018). However, this “subsetization” is still under debate as it was demonstrated in mice by *Abbas et al.* that pDC’s phenotypes and functions may vary overtime after MCMV infection (Abbas et al. 2020). Using velocity, they showed that pDCs first start to produce IFN-I once triggered, then transition towards T cells activating functions. Nevertheless, similar studies still need to be performed on human tumor associated (TA)-pDCs to understand all of their functions.

### **D. Mature DC**

Mature DCs could be defined as a terminally differentiation state of DCs linked with specific phenotypic changes, with a high expression of co-stimulatory molecules (CD80, CD86), and the upregulation of maturation markers such as LAMP3 (DC-LAMP/CD208) (Ladányi, Kiss, Somlai, Gilde, Fejős, et al. 2007). Recently, a DC subset named mreg-DCs was identified by scRNA-seq of immune cells present in non-small cell lung carcinoma NSCLC. These mreg-DCs display high levels of CD40, CD80, CD86, CD83, PD-L1, PD-L2 genes, but more importantly, mreg-DCs highly expressed DC-LAMP transcripts, suggesting they may represent a subset of mature DCs (Maier et al. 2020). Actually, mregDC express a transcriptomic signature of activated DC. It has been demonstrated that mature DC represent a convergence of cDC1 and cDC2 which lost their respective surface marker expression and reach a final stage of maturation in tissue (J. Qian et al. 2020; Cheng et al. 2021).

## E. Other DC subsets

### 1. MoDCs

In inflammatory contexts, monocytes can differentiate into inflammatory DCs (Inf-DCs), also called MoDCs (León, López-Bravo, and Ardavín 2005; 2007). MoDCs share surface markers with cDC2, as they can be identified by flow cytometry as HLA-DR<sup>+</sup> CD11c<sup>+</sup> BDCA1<sup>+</sup> SIRP $\alpha$ <sup>+</sup>. In some tissues, they can express low level of CD14 and Fc $\epsilon$ R1. To date, there is no specific marker to distinguish *in situ* cDC2 from MoDC in humans. MoDCs can respond to danger signals, act as APCs in tissues and induce T cell responses (Bakdash et al. 2016), making them important immune cells in anti-tumoral responses.

### 2. Axl-DC

AS-DC or Axl-DC subset, was described within the lineage- HLA-DR<sup>+</sup> population as a AXL<sup>+</sup> Siglec-6<sup>+</sup> CD34<sup>+</sup> cells (Villani et al. 2017). They are actually thought to be pre-DCs as they can differentiate either into cDC1 or cDC2 and activate T cells vigorously after activation (See et al. 2017). Furthermore, *Alcántara-Hernández et al.* demonstrated that only half of Axl-DC expressed CD11c, while they all express CD123 and BDCA4, thus potentially leading to the contamination of the pDCs fraction by Axl-DC during pDCs sorting (Alcántara-Hernández et al. 2017). This observation supports the hypothesis that Axl-DC are a pre-DC subset and should be taken into consideration during DC subsets sorting by flow cytometry with conventional gating strategy.

As Axl-DC do not express BDCA1 nor BDCA3 markers (Alcántara-Hernández et al. 2017), it is possible that Axl-DC belong to the so-called “double negative” population present in tumors when analyzed by flow cytometry. Indeed, in some gating strategies used to observe DCs in the TME, pDCs are first isolated as CD11c<sup>-</sup> CD123<sup>+</sup> or BDCA2<sup>+</sup> cells from the lineage- HLA-DR<sup>+</sup> gate. Then CD11c<sup>+</sup> CD123<sup>-</sup> cells are separated into cDC1 and cDC2 thanks to their respective expression of BDCA3 or BDCA1, leaving a major unstudied population of lineage- HLA-DR<sup>+</sup>, CD11c<sup>+</sup>, BDCA1<sup>-</sup> BDCA3<sup>-/low</sup> cells representing around 80% of the DCs. Thus, Axl-

DC could be present in the “double negative” population, and anti-Axl antibody should be added to flow cytometry panels in order to further investigate the role of Axl-DC in anti-tumor response.

## **II. Role of DC subsets in the anti-tumoral immune response**

### **A. The antitumor function of cDC1**

Even though the roles of DC subsets are well characterized in infectious contexts, the respective function of each DC subset within the TME still needs to be clearly established. Here, we will review the functions of the different DC subsets within the TME and identify the advantages and caveats of different high-throughput technologies that could be used to predict their role in the antitumoral immune response, but these observations would have to be confirmed *in vitro* or *in vivo*. For instance, RNA-seq enables the deconvolution of immune cell type infiltration and thus calculation of specific infiltration scores. In this context, it was shown that a high tumor infiltration in myeloid cells, but not in total immune cells, was associated to the responsiveness to anti-PD-1 immunotherapies (Barry et al. 2018). More precisely, an increased proportion of cDC1 among myeloid cells strongly predicted a good response to immunotherapies, while cDC2 infiltration had no impact on the patient’s responsiveness. Therefore, the authors showed that tumors not only have to be highly infiltrated with immune cells, but it has to be highly infiltrated with specific populations such as cDC1 to respond to immunotherapies. It is now known that these good prognosis and good response to immunotherapies following a high infiltration of cDC1, are possible thanks to their capacity to cross-presentation, communicate with NK cells and to secrete type III interferon (IFN-III). These three major anti-tumor functions of cDC1 will be detailed here.

### **3. cDC1 Antigen cross-presentation**

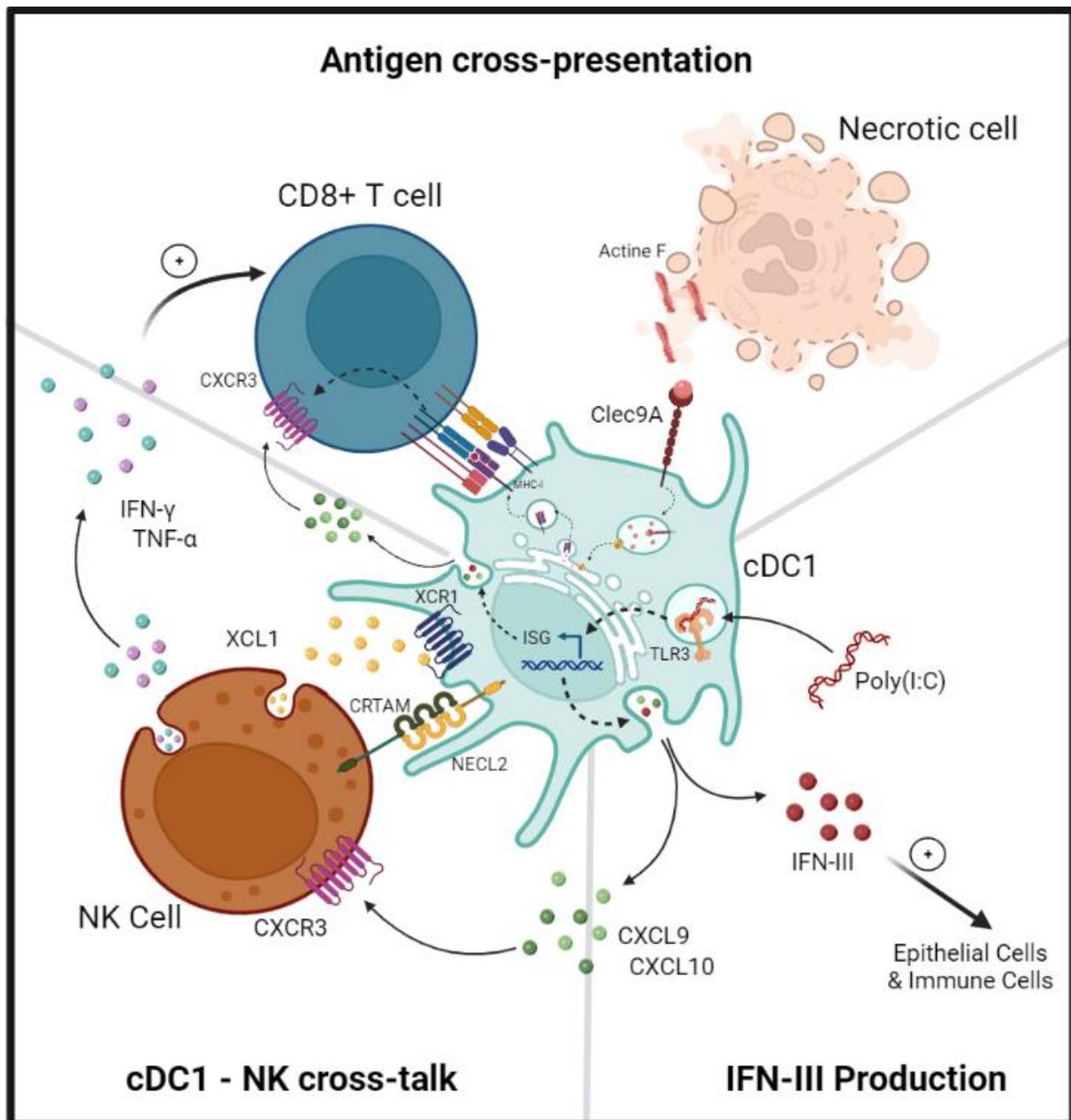
Given their ability to activate cytotoxic immune responses through antigen cross-presentation, the role of cDC1 in anti-tumor immunity has been extensively investigated in mice. It was shown that cDC1 have the capacity to engulf tumor necrotic cell fragments (Broz et al. 2014), and were described to be the unique cell type to transport antigens from the tumor site into the lymph nodes to prime CD8+ T cells (Salmon et al. 2016). Mice lacking cDC1s have less tumor-specific CD8+ T cells, hence an impaired antitumor response (Hildner et al. 2008; Lionel F. Poulin et al. 2012; Hammerich et al. 2019; Maier et al. 2020; Mattiuz et al. 2021). In humans, only few papers were published about cDC1 functions in anti-tumor response. Still, their presence in the tumor has been proven to be positively associated with cancer patient survival (A. R. Sánchez-Paulete et al. 2017; Hubert et al. 2020; Wculek et al. 2020; Broz et al. 2014) and associated to a good response to checkpoint blockade immunotherapy (Wculek et al. 2020), making cDC1 ideal candidates for the development of immunotherapies that mobilize CD8+ T cells against cancer. Nevertheless, the capacity of human cDC1 to cross-present antigen within human tumors remains to be confirmed.

#### **4. cDC1 cross talk with NK cells**

In humans, the levels of TA-NK and TA-cDC1 genes correlate with clinical positive outcome in melanoma patients (Barry et al. 2018; Böttcher et al. 2018; Dikshit et al. 2020). The ligands *CCL5*, *XCL1*, and *XCL2* genes were also enriched and were described as main actors of the crosstalk NK-cDC1 in mice (Bödder et al. 2021). Moreover, in the tumor the expression of NECL2/CADM1 lectin and CRTAM by cDC1 and NK cell respectively, also implies a cDC1/NK cell cross talk (Galibert et al. 2005; Arase et al. 2005; Boles et al. 2005). These observations highlight a bidirectional cross-talk between NK cells and cDC1 in tumors (Peterson and Barry 2020; Bödder et al. 2021). In this context, our team demonstrated that the production of TNF- $\alpha$  and IFN- $\gamma$  by NK cells potentializes cDC1 antigen cross-presentation to CD8+ T cells (Deauvieu et al. 2015). NK cells also produce the FMS-like tyrosine kinase 3 ligand (FLT3L), which stimulates cDC1 survival, differentiation, and recruitment, thus enhancing the accumulation of cDC1s in the TME (Barry et al. 2018).

## 5. IFN-III secretion by cDC1

A unique property of cDC1 is to produce elevated amounts of IFN-III during infectious diseases or after TLR3 engagement (Lauterbach et al. 2010; Yoshio et al. 2013; Balan et al. 2014). In the context of anti-tumour response, our team recently demonstrated that i) the production of IFN-III in breast cancer was restricted to cDC1, and ii) the presence of IFN-III in the TME correlated with a favorable clinical outcome (Hubert et al. 2020). We further described a strong induction of IFN-III production by cDC1 upon TLR3 stimulation with Poly(I:C) in breast tumors that correlates with the increased secretion of chemokines, such as CXCR3-L (CXCL11/CXCL10/CXCL9) and CX3CL1, as well as the increased secretion of cytokines, like TNF- $\alpha$  and IL-12p40. Thus, we uncovered the potential of Poly(I:C)-stimulated cDC1 to induce a Th1-related immune response in breast TME, accounting for the recruitment and activation of cytotoxic effector cells (CD8+ T cells and NK cells) (Hubert et al. 2020).



**Figure 2 –Antitumor functions of cDC1**

Antigen Ag cross-presentation: cDC1 superiority at antigen cross-presentation is due to their expression of CLEC9A that binds to Actine F from necrotic cancer cells and allow the internalization of cellular fragment. These fragments will be processed to become tumor associated antigens (TAA) presented by the MHC-I to CD8+ T cells. IFN-III production: Upon activation through TLR3 with Poly(I:C), TA-cDC1 will induce the production of IFN-III that will be secreted in the TME and will impact the activation state of other epithelial and immune cells. cDC1 – NK cells cross-talk: At the same time as the production of IFN-III, TA-cDC1 will also start the transcription of Immune Stimulated Genes (ISG) leading to the synthesis and secretion of CXCL9 and CXCL10. Both chemokines bind CXCR3 receptor involved in the recruitment of NK cells and CD8+ T cells toward cDC1 and enhance their activation. To ensure an interaction between cDC1 and NK cells, NK cells produce XCL1 which will bind XCR1 receptor present at cDC1 membrane supporting a cross-talk between these two cells types. Once localized in the same area, NK cells can interact with cDC1 through CRTAM and NECL2 pathways. Finally, activated NK cells produce large quantities of IFN-γ and TNF-α involved in CD8+ T cell activation.

## **B. The antitumor properties of tumor-associated cDC2**

Despite their high heterogeneity and their capacity to induce a broad spectrum of immune responses, very little is known about cDC2's role in the anti-tumor response. It is only recently that an elegant paper published in 2019, focused on cDC2 heterogeneity in the context of head and neck squamous cell carcinoma (HNSCC). Using flow cytometry analysis in 32 human HNSCC samples, the authors demonstrated that cDC2 were essential for generating antitumor CD4+ T cell responses (Binnewies et al. 2019). The balance between cDC2 and regulatory T cells (Tregs) frequencies could also play an important role in the quality of T cell responses and in patients' prognosis in HNSCC (Binnewies et al. 2019). In 2020, a second paper used multispectral immunofluorescence and flow cytometry to study Human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC) (Santegoets et al. 2020). The authors showed that TA-cDC2 can stimulate CD4+ T cell and induce a Th1 polarization, thanks to the production of IL-12 (at a lesser extent than cDC1) and IL-18. Moreover, they demonstrated a positive correlation between a high infiltration of cDC2 and tumor-specific T cells within the tumor, which was linked to a prolonged survival. Finally, *Michea et al.* performed RNA-seq on DCs sorted from human primary breast cancer and healthy mammary tissue (Michea et al. 2018). They identified 490, 88, 40, and 4 differentially expressed genes (DEG) specifically upregulated in TA-pDCs, TA-cDC1s, TA-MoDCs, and TA-cDC2s compared to healthy tissue, respectively. cDC2 is the subset with the highest heterogeneity, thus the identification of a unique gene expression signature for cDC2 within the TME is more difficult. The authors also reported that a cDC2 enrichment, assessed by TA-DC subtype gene signatures, correlated with a better survival in human breast cancer patients suggesting that enhancing the cDC2 - CD4+ T cell axis may represent an effective strategy to treat breast cancers. All together, this data highlights the important role of intra-tumoral cDC2 to stimulate tumor-infiltrating T cells and exert their anti-tumoral properties. Nevertheless, cDC2 heterogeneity remains to be fully deciphered to understand their exact features and contributions to the anti-tumoral response.

### **C. Mature DC contribute to TLS formation and have anti-tumor function**

On tumor sections, DC-LAMP+ mature DCs are commonly observed in clusters (Movassagh et al. 2004), close to lymphocytic infiltrates (Ladányi, Kiss, Somlai, Gilde, Fejős, et al. 2007). They are found in the CD3+ T cell zone surrounding CD20+ B cell follicles and germinal centers, also called tertiary lymphoid structures (TLS) formed at the tumor periphery (Dieu-Nosjean et al. 2008; Sautès-Fridman et al. 2011). Therefore, DC-LAMP+ DCs are classically used as a surrogate marker for TLS in tumors, and the presence of such TLS within tumors is associated with a good clinical outcome in early stage NSCLC (Dieu-Nosjean et al. 2008) and 10 other types of cancers (Sautès-Fridman et al. 2016).

In ovarian cancer, an important Th1 polarization and cytotoxic activity was strongly associated with a high density of TA-DC LAMP+ DCs (Truxova et al. 2018), which recall the specific cDC1 capacity to induce a Th1 polarization and cytotoxic activity. This polarization toward Th1 could be linked to the high level of *IL12B* RNA in mature DC (Cheng et al. 2021). In addition, *Zillionis et al.* identified a DC cluster by scRNA-seq in human NSCLC, corresponding to DCs expressing DC-LAMP and BATF3, a transcription factor involved in the cDC1 differentiation. However, this cluster completely lacks the expression of CLEC9A or XCR1 (Zillionis et al. 2019). In a study by *Qian et al.*, authors used pseudo-time analysis on 2,722 sorted DCs from 3 different cancer types (ovarian, lung and colon) and were able to relate mature DC with other DC subsets. Mature DCs were shown to derive mostly from cDC2 (J. Qian et al. 2020). During this maturation process CLEC10A expression was down regulated while the expression of BIRC3, which encodes a multi-functional protein regulating not only caspases and apoptosis, but also inflammatory signaling (NFkB pathways), was increased. In contrast *Zhang et al.* have shown in hepatocellular carcinoma that both cDC1 and cDC2 can transform into mature DCs (Qiming Zhang et al. 2019). Moreover, mreg-DCs that could be related to mature DC as they express high level of DC-LAMP transcript, display a very different transcriptomic profile compared to cDC1 and cDC2 (Maier et al. 2020) even though their results suggest that cDC1

and cDC2 both contribute to the mreg-DC subset. These results suggest that, in the TME, the mature subset is composed of cDC1 and cDC2 that underwent transcriptional changes toward a mature DC transcriptional state even if some of their lineage-intrinsic features are conserved in mature state (Kvedaraite and Ginhoux 2022).

#### **D. MoDCs and DC3 subsets characterization and functions in anti-tumor response**

Monocytes differentiate into moDC in inflammatory tissue, but it has also been shown that monocytes can differentiate into MoDCs at the tumor site (B.-Z. Qian et al. 2011; Shand et al. 2014) and have been reported in lung and colorectal cancers (Alfonso R. Sánchez-Paulete et al. 2016), in melanoma draining lymph nodes, as well as in HNSCC tumors (Binnewies et al. 2019). Even if further studies must be performed in order to fully comprehend their function in the TME, it has been demonstrated that MoDCs from the inflammatory environment of ovarian tumor ascites are able to induce CD4<sup>+</sup> T cells polarization towards Th17 (Segura et al. 2013). Recently, RNA-seq analysis of DCs performed by *Bourdely et al.* allowed the identification of a potential new DC subset called DC3 within the breast TME, which could be related to moDCs. This new subset is characterized as CD14<sup>low</sup> CD1c<sup>+</sup> CD163<sup>+</sup> CD88<sup>-</sup> DC-Like cells. It was proposed to be an intermediate population between cDC2 and MoDC, as they can prime naïve T cells and secrete IL-12p70 and IL-23 like cDCs as well as TNF- $\alpha$  like monocytes and DCs. Tumor infiltration by this particular DC3 population is positively correlated to a Trm infiltrate (CD8<sup>+</sup> CD103<sup>+</sup> CD69<sup>+</sup> T cells) which was not the case with other mononuclear phagocytes (Bourdely et al. 2020).

## **E. LC and their numerous functions in tumor regression and their response to immunotherapies**

LCs have been identified in numerous skin cancers (Shevchuk et al. 2014), but also in breast (Tsuge, Yamakawa, and Tsukamoto 2000), prostate (Bigotti, Coli, and Castagnola 1991), gastric (Tsujiyama et al. 1987), head and neck (Kindt et al. 2016), and cervical (Manickam, Sivanandham, and Tourkova 2007) cancers. In cervical cancer patients treated with radiotherapy, a high LCs infiltration was associated with a strong T cell infiltration and a good prognosis (Nakano et al. 1992). The role of LCs was nicely reviewed by *A. Rajesh and M. Hibma* highlighting their capacity to regulate immunity, carcinogenesis, lymphangiogenesis, and angiogenesis in the TME (Rajesh and Hibma 2020). Unexpectedly, it was also shown that migrating LCs start to express the programmed cell death protein 1 (PD-1) receptor which reduced their capacity to activate T cells. The co-culture of PD-1+ LC with allogenic CD4+ T cells showed a significantly decreased CD4+ T cell alloresponse that was not observed when cultivated with anti-PD-1 antibody. Thus, using anti-PD-1 antibody as an immunotherapy treatment to block PD-1 on LCs, as well as on T cells, could be beneficial for cancer patients (Peña-Cruz et al. 2010).

## **F. In situ visualization a tool to link tumor associated-DCs infiltration and their impact on prognosis**

It has long been demonstrated thanks to multiplex immunofluorescence (mIF) that cancer prognosis varies with i) the infiltration of a specific DC subset and ii) with the type of cancer as shown in **[Table1]**. Our team published a protocol paper to visualize and analyze DC *in situ* using mIF. For instance, cDC1 infiltration in the TME correlated with a positive prognosis in breast cancer (Böttcher et al. 2018; Michea et al. 2018), melanoma (Böttcher et al. 2018; Barry et al. 2018), Head and Neck cancer (Böttcher et al. 2018), lung cancer (Böttcher et al. 2018), and in 12 other types of cancer (Broz et al. 2014). However, cDC2 infiltration in the TME correlated with a positive prognosis in breast (Hillenbrand, Neville, and Coventry 1999;

Iwamoto et al. 2003; Coventry and Morton 2003) and melanoma (Ladányi, Kiss, Somlai, Gilde, Fejos, et al. 2007) cancers, but cDC2 infiltration can also correlate with a negative prognosis in lung (Tabarkiewicz et al. 2008) and colorectal cancer (Sandel et al. 2005), and has no influence on the prognosis of HNSCC (Goldman et al. 1998) and renal cell cancer (RCC) (Schwaab et al. 1999). Similar observations were made for mature DCs infiltration that was found to be associated with a positive prognosis in melanoma (Movassagh et al. 2004; Ladányi, Kiss, Somlai, Gilde, Fejos, et al. 2007), lung (Dieu-Nosjean et al. 2016) and breast cancer (Martinet et al. 2013), but with a negative one in colorectal (Sandel et al. 2005) and stomach (Ishigami et al. 2000) cancers. Regarding pDCs, their infiltration in the TME has a positive or a negative impact of patients` prognosis depending of the cancer type (Michea et al. 2018; Sisirak et al. 2012; Labidi-Galy et al. 2012; Treilleux et al. 2004; Aspod et al. 2013; Labidi-Galy et al. 2011; Oshi et al. 2020; Kießler et al. 2021).

Nevertheless, the main limitation with mIF technic is the low number of colors available explaining why the use of other technologies such as Imaging Mass Cytometry (IMC) are increasing. Indeed, thanks to the use of antibody linked to heavy metals to stain FFPE and frozen tissues, *R.Elaldi et al.* were able to analyze tissue section of squamous Cell Skin Cancer (cSCC) with 39 antibodies to identify a large panel of immune cells (Elaldi et al. 2021). More precisely, CD11c, HLA-DR, DC-LAMP, CD207, DC-SIGN, CD16, and CD14 were used to identify many DC subsets (Ijsselsteijn et al. 2019). Altogether, the data they have generated provide valuable information on the localization of DC subsets within cSCC tissues.

**Table 1 DC Subsets prognosis in different cancer types**

Population	Impact on prognosis	Tumor Type	References
cDC1s	Positive	Breast	<i>Bottcher et al. Cell 2018</i>
			<i>Michea et al, Nature Imm 2018</i>
		Melanoma	<i>Bottcher et al. Cell 2018</i>
			<i>Barry et al. Nature Med 2018</i>
		Head and neck	<i>Bottcher et al. Cell 2018</i>
		Lung	<i>Bottcher et al. Cell 2018</i>
		PDAC	<i>Plesca et al, Cancers 2022</i>
12 other cancers	<i>Broz et al. Cancer Cell 2014</i>		
cDC2s	Positive	Breast	<i>Hillenbrand et al. Br J Cancer 1999</i>
			<i>Iwamoto et al. Int J Cancer 2003</i>
			<i>Coventry and Morton Br J Cancer 2003</i>
		Melanoma	<i>Ladanyi et al. Cancer Immunol 2007</i>
	Head and neck	<i>Santegoets SJ et al. J Immunother Cancer 2020</i>	
	Negative	Lung	<i>Tabarkiewicz et al. Oncol Rep 2008</i>
		Colon	<i>Sandel et al. Clin Cancer Res 2005</i>
	No impact	Breast	<i>Treilleux et al. Clin Cancer Res 2004</i>
Kidney		<i>Schwaab et al. J Urol 1999</i>	
Head and neck		<i>Goldman et al. Arch Otolaryngo 1998</i>	
pDCs	Negative	Breast	<i>Treilleux et al. Clin Cancer Res 2004</i>
			<i>Sisirak et al. Cancer Res 2012</i>
		Ovary	<i>Labidi-Galy et al., Oncoimmunol 2012</i>
			<i>Labidi-Galy et al. Cancer Res 2011</i>
	Positive	Breast	<i>Michea et al, Nature Imm 2018</i>
			<i>Oshi et al, Cancers 2020</i>
		Colon	<i>Keissler et al, JITC 2021</i>
PDAC	<i>Plesca et al, Cancers 2022</i>		
No impact	Melanoma	<i>Aspord et al. Cancer Immunol Res 2013</i>	
mature DCs	Positive	Melanoma	<i>Ladanyi et al. Cancer Immunol Immunother 2007</i>
			<i>Movassagh et al. Cancer Res 2004</i>
		Lung	<i>Dieu-Nosjean et al., J Clin Oncol 2008</i>
	Breast	<i>Martinet et al., J Immunol 2013</i>	
	Negative	Colon	<i>Sandel et al. Clin Cancer Res 2005</i>
		Stomach	<i>Ishigami et al. Clin Cancer Res 2000</i>
No impact	Breast	<i>Treilleux et al. Clin Cancer Res 2004</i>	

### III. Focus on pDCs and their function in the TME

pDCs can migrate directly from blood to lymph node through high endothelial venules (HEV) thanks to their expression of PSGL1, CXCR4 (Kohrgruber et al. 2004), CCR7 (Umemoto et al. 2012), CD62L, CCR5 and CXCR3 (Sozzani et al. 2010). pDCs can also infiltrate inflamed tissues as they express CXCR3, CCR2, CCR5, CCR6, and CCR10 (Tiberio et al. 2018). Interestingly, it has been demonstrated that the stromal-derived factor-1 (SDF-1)/CXCL12 – CXCR4 axis was involved in the recruitment of pDCs in ovarian tumors (Zou et al. 2001). It appears that ovarian tumor cells secrete CXCL12, which can be found in tumor supernatants and ascites (Tel, Sittig, et al. 2013). The authors performed chemotaxis assay and transmigration assay where pDCs exposed to tumor supernatants, ascites or CXCL12 had an important migration compared to the conditions where anti-CXCR4 antibody was added to the culture. In melanoma, pDCs recruitment within the TME could be induced by the CCL20/CCR6 axis (Charles et al. 2010)

pDCs infiltrate tumor but their role in the TME is very controversial as they have been associated with poor and good prognosis in different cancers [**Table 1**], the same is true for pDC functions in the TME. As reviewed below, different studies have demonstrated that pDCs do have immunostimulatory properties thanks to their antigen (cross-) presentation, and their direct cytotoxic capacities, along with their production of IFN-I [**Figure 4**]. However, different evidences show that pDCs can be inhibited in the TME and can cause Tregs expansion.

#### A. Antigen presentation properties

After infiltrating tumors, antigen uptake of Tumor-Associated Antigen (TAA) by pDCs is key for the activation of a specific antitumor immune response. pDCs express a range of proteins involved in antigen uptake such as DEC-205, DCIR, BDCA2 and CD32 (Benitez-Ribas et al. 2008; Meyer-Wentrup et al. 2008; Tel et al. 2011), but it has been demonstrated that BDCA2 was the receptor inducing the highest percentage of antigen uptake (Tel et al. 2011). It is important to acknowledge that only 45% of pDCs indeed uptake antigens in this study.

In mice Siglec-H expression is characteristic of pDCs (A. L. Blasius et al. 2006) in blood, bone marrow, spleen, LN, liver, and thymus (A. Blasius et al. 2004). Siglec-H was associated with antigen capturing functions. Indeed, after interaction with OVA-coated antibody, OVA is internalized and processed leading to cross-presentation to CD8+ T cells (J. Zhang et al. 2006). It was also demonstrated that Siglec-H is involved in pathogen endocytosis such as porcine arterivirus (Delputte and Nauwynck 2004; Fuchs et al. 2005). Interestingly, one of Siglec-H human counterpart is Siglec-5. Siglec-5 is expressed by human pDCs, interact with anti-Siglec-5 antibodies and induce their rapid uptake into the early endosome (Lock et al. 2004). Siglec-5 is also involved in the recognition and phagocytosis of *Neisseria meningitidis* bacteria (Jones, Virji, and Crocker 2003). Several studies also reported antigen presentation capacity for pDCs (Grouard et al. 1997; M. Cella et al. 2000). Indeed, it was demonstrated that activated pDCs with tetanus toxoid or nanoparticles containing tetanus toxoid can perform antigen presentation and induced CD4+ T cell proliferation (Tel, Sittig, et al. 2013). Furthermore, human pDCs were cultivated with gp100<sub>272-300</sub> long peptide, a melanoma-associated tumor peptide, and were able to process it into smaller gp100<sub>280-288</sub> peptide and upload it on their MHC-I to present it to gp100<sub>280-288</sub> specific CD8+ T cells. Similar results and conclusion were drawn in another experiment (Salio et al. 2003). Even though pDCs are indeed capable of antigen (cross-) presentation, they do antigen cross-presentation at a lower extent than cDCs (Tel, Schreibelt, et al. 2013).

Altogether, it has been well reported that pDCs can infiltrate tumors, can uptake TAA and activate specific adaptive immune responses *ex vivo* through antigen (cross-) presentation. Still, we are lacking evidence of human TA-pDCs up taking and processing TAAs, before to migrate to the draining lymph node to elicit a strong specific immune response.

## **B. Direct cytotoxic properties**

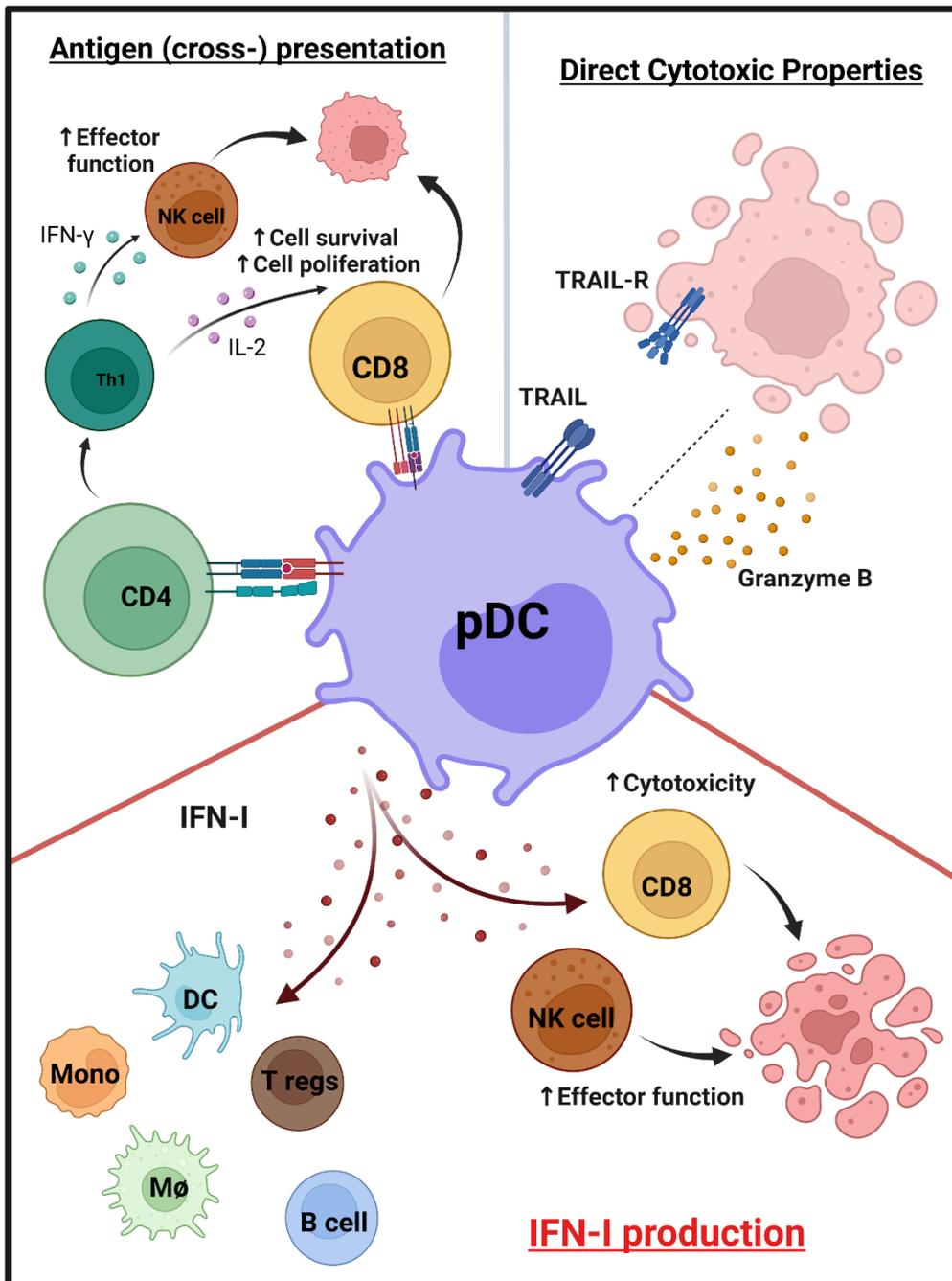
Another interesting property of pDCs is their ability to secrete Granzyme B (GrzB) and express Tumor-necrosis-factor related apoptosis inducing ligand (TRAIL) after TLR activation. It has been demonstrated that upon Influenza infection or through the use of TLR7 or TLR9 agonist pDCs will express TRAIL (Chaperot et al. 2006). pDCs could lyse melanoma cells after stimulation with CpG ODN (TLR9 agonist) or R848 (TLR7 and TLR8 agonist) (Stary et al. 2009). HIV infection turns pDCs into TRAIL-expressing IFN-producing killer pDC (IKpDC) by triggering their TLR7 receptor (Hardy et al. 2007b). Interestingly, Tel et al reported that CD56 upregulation, a marker commonly expressed on cytotoxic lymphocytes (NK and NKT cells), correlated with increased expression of GrzB and TRAIL on pDCs (Tel et al. 2012). However, they demonstrated that cell lysis was due to a cell-to-cell contact, which support an important role of TRAIL over GrzB in pDCs cytolytic properties. Another study also observed an expression of GrzB and TRAIL on pDCs activated with Imiquimod (IMQ) (Kalb et al. 2012). To ensure that pDCs could lyse cells they performed cytotoxic assay with IMQ activated pDCs and SKMel2 and WM793 melanoma cell lines. After 4h of culture around 60% and 50% of specific lysis was observed with SKMel2 and WM793 respectively. They performed the same experiment but pDCs were first pre-treated with anti-TRAIL antibody showing a clear involvement of TRAIL in a cell-to-cell manner. Importantly they also show that pDCs pre-treatment with IFN- $\alpha$  was necessary for an optimal cell lysis through TRAIL. Finally, BCC treatment with IMQ induced direct cytotoxic properties in TA-pDCs (Stary et al. 2007)

## **C. Type I Interferon production**

Even if pDCs have antigen presentation and cytotoxic properties, they are rather known for their abilities to secrete huge amounts of IFN-I. This secretion of IFN-I is highly considered as it can have several immunostimulatory effects on many immune cells (Gresser and Belardelli 2002; Zitvogel et al. 2015). Moreover, IFN-I have a direct effect on cancer cells thanks to its anti-proliferative and pro-apoptotic functions (see. Chapter II, IV. 1.). These observations led

to the approval of IFN-I, more precisely IFN- $\alpha$ 2A and IFN- $\alpha$ 2B, as a treatment of several malignancies (Asmana Ningrum 2014). An IFN-I signature in the TME correlates with “hot tumors” phenotype that is characterized by a strong immune infiltrate and a better responsiveness to immunotherapies. Conversely, the downregulation of IFN-I receptor (IFNAR1) is associated with an adverse clinical outcome and an increased tumor progression (Katlinski et al. 2017; Castiello et al. 2018) in colorectal carcinoma (Katlinski et al. 2017) and melanoma (Sprooten, Agostinis, and Garg 2019). IFN-I can enhance innate and adaptive immune response. Bencze *et al.* nicely reviewed IFN-I impact on innate cells such as monocyte, macrophages, DCs and NK cells (Bencze, Fekete, and Pázmándi 2021), with NK cells being of great interest in antitumor response due to their direct cytotoxic activities leading to cancer cell destruction. IFN-I secretion will enhance NK cell activation, cytotoxicity, and is involved in their proliferation (Swann et al. 2007). Concerning adaptive immune cells, IFN-I will facilitate Th1 differentiation, help B cell activating capacity, but IFN- $\alpha$  can also be inhibit Treg expansion. Our lab demonstrated that impaired IFN-I production by human TA-pDCs favours Tregs expansion (Sisirak et al. 2012). IFN-I also increase B cells activation, class switching, plasmablast differentiation, and antibody production of plasma cells (Bencze, Fekete, and Pázmándi 2021). The last adaptive immune cells that is significantly affected by IFN-I stimulation are CD8<sup>+</sup> T cells. IFN-I increase CD8<sup>+</sup> T cells clonal expansion, survival (Bencze, Fekete, and Pázmándi 2021), but more importantly it increases their IFN- $\gamma$  production along with their cytotoxicity (Curtsinger, Johnson, and Mescher 2003; Curtsinger et al. 2005; Mescher et al. 2006).

Overall, IFN-I are key important cytokines in the TME as it has such a tremendous impact on innate and adaptive immune cells. Actually, as IFN-I have incredible immunostimulatory properties, it explains why pDCs, that are the main producers of IFN-I, are deeply studied in the TME. Indeed, if there is a function that we want to maintain or enhance in TA-pDC, it is their IFN-I production as it could favor an immunostimulatory microenvironment instead of an immunosuppressive one.



**Figure 3 – pDC immunostimulatory functions**

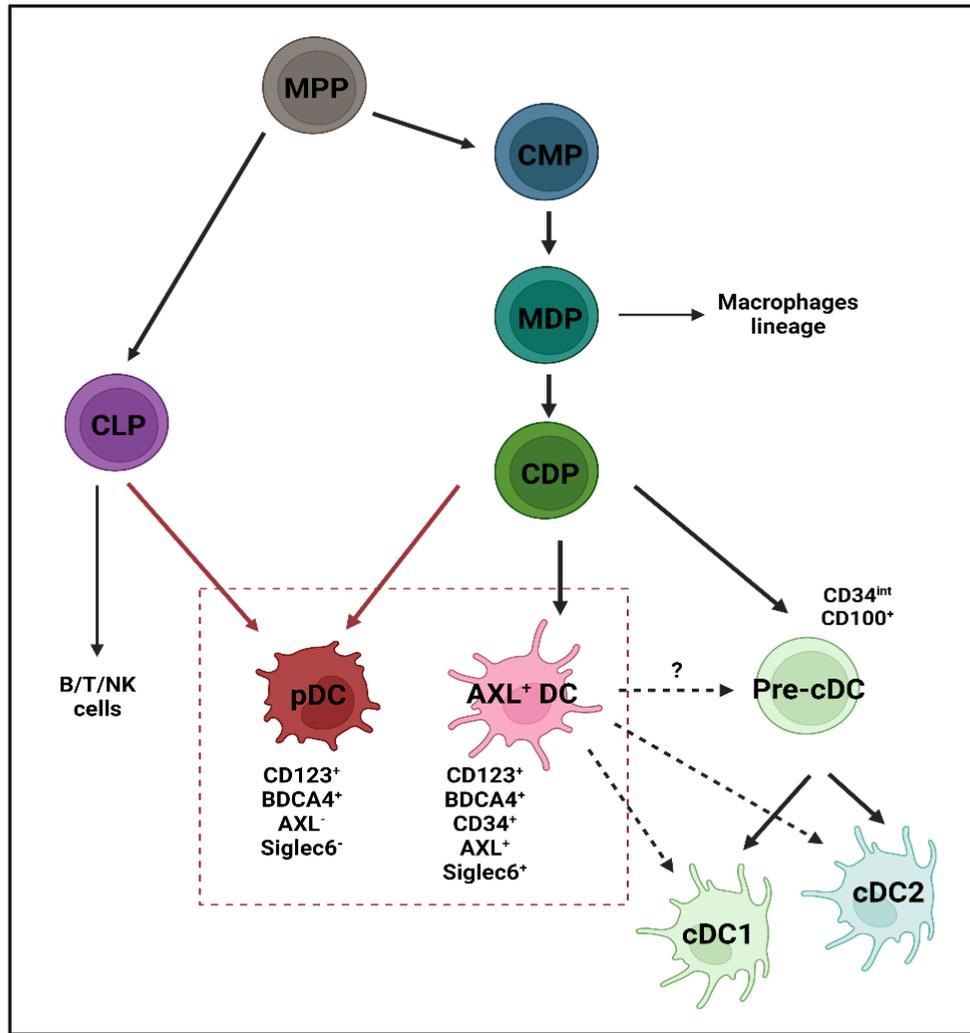
Upon the appropriate activation signal pDCs can perform antigen presentation to CD4+ T cells, activate them and induce Th1 polarization. These Th1 CD4+ T cells will secrete IFN- $\gamma$  that will enhance NK cell effector functions involved in cancer cell lysis. Th1 CD4+ T cells will also secrete IL-2 which is necessary for CD8+ T cells survival and proliferation. pDCs can also perform antigen cross-presentation to activate CD8+ T cells, which are important in tumor regression. If pDCs are stimulated with FSME or TLR7/8 ligands, they will start to produce Granzyme B, inducing apoptosis in the targeted cells. But they will also upregulate their expression of TRAIL. In cell-to-cell interaction TRAIL will interact with its receptor TRAIL-R expressed by cancer cell, inducing apoptosis. Finally, after stimulation pDCs will secrete high quantities of IFN-I that will have an immunostimulatory effect of innate immune cells such as DC, monocyte, Macrophages (M $\phi$ ), and NK cells. IFN-I will increase NK cell effector functions leading to cancer cell destruction. IFN-I produced by pDCs will also impact adaptive immune cells such as B cell, Tregs and CD8+ T cells. CD8+ T cells will have a cytotoxic enhancement after IFN-I stimulation.

## **D. pDC heterogeneity**

As only a small fraction of pDCs can do antigen-presentation or can specialize in IFN-I production, it may seem that there is a heterogeneity in pDCs functions. This heterogeneity raises the question of fully differentiated pDCs that restrain plasticity to adapt to different pathogen or damage. Or rather pDCs different ontogeny creates different pDC subsets with different functions.

### **1. pDCs lineage and functional heterogeneity**

In humans, it has been reported that pDCs could be generated from common DC progenitors (CDP) and from the common lymphoid progenitor (CLP) at steady state (Rodrigues et al. 2018) [Figure 3]. The fact that pDCs derived from two different lineages could contribute to their functional heterogeneity. In order to compare CDP-derived pDCs and CLP-derived pDCs function, *Yang et al.* performed adoptive transfer of these two sets into wild type mice to follow their differentiation and evaluate their functions (Yang et al. 2005) . It appeared that CDP-derived pDCs were better at secreting IFN-I and inducing T cell activation and IFN- $\gamma$  production after CpG stimulation, compared to their CLP counterparts. Nevertheless, similar observations are lacking in human context due to the difficult access to equivalent samples. Several studies did describe T cell activation potential in pDCs (Grouard et al. 1997; M. Cella et al. 2000; Hoeffel et al. 2007). But their results could be debated as pDCs are commonly cultured with IL-3 and/or CD40L, which can induce a bias in their observations, and because of a probable contamination with AXL+ DC subsets. Indeed, as Axl+ DC subset all express CD123 and BDCA4/CD303, with a half of their population expressing CD11c at their surface, they can easily be sorted along with pDCs (Villani et al. 2017). Still, Axl+ DCs can vigorously activate T cells right after they differentiated into cDC1 or cDC2 (See et al. 2017). These new results put in perspective all previous work showing antigen presentation capacity of pDCs, as this function could be attributed to AXL+ DC instead of pDCs. For further pDC functional analysis, researchers shall ensure AXL+ DC exclusion from their sorting strategy.



**Figure 5 – pDC lineage in humans**

As a result of external signals, multipotent progenitors (MPP) can differentiate into common myeloid progenitors (CMP), followed by a differentiation into monocyte and DC progenitors (MDP) before becoming common DC progenitors (CDP). CDP restrain the capacity to become either i) pre-cDC cell, leading to fully differentiated cDC1 or cDC2, ii) AXL<sup>+</sup> DCs, or iii) fully differentiated pDCs. From a second lineage pDCs can be generated from common lymphoid progenitors (CLP). The border dashed red square indicates the potential mischaracterization of AXL<sup>+</sup> DCs as pDCs.

## 2. pDCs subpopulations and their different functions

In the context of viral infections, it has recently been suggested that there may be subpopulations within the pDC subset. Indeed, it has been proposed by *Alculumbre et al.* that 3 subpopulations of human pDCs could be identified with different proportions depending on the type of stimulus received by non-activated pDCs. These 3 subpopulations can be differentiated by their expression of PD-L1 and CD80 (Alculumbre et al. 2018). PD-L1+ CD80– pDCs, named P1 subpopulation, displayed a plasmacytoid morphology and specialization in IFN-I secretion. P3 subpopulation, characterized as PD-L1- CD80+, have a dendritic morphology and are able to present antigen to activate CD4+ T cells. The last subpopulation P2 which are PD-L1+ CD80+, appears to be an intermediate population able to secrete IFN-I and to activate CD4+ T cells, both functions at lesser extent. These results support previous observations where only a small fraction of pDCs were stained positive in flow cytometry for IFN- $\alpha$  production. This observation could have been explained by the use of a single anti-IFN- $\alpha$  antibody at a time where it exists 12 different IFN- $\alpha$  that could be produced by the other pDCs.

However, with the accumulating evidences of pDCs heterogeneity, a very recent study performed sc-RNA sequencing on human pDCs stimulated with Influenza virus during 24h (Ghanem et al. 2022). They demonstrated that only 1 cluster out of the 4 identified cluster where specialized in IFN-I production, with a second cluster with a lower expression of TCF4 but a higher expression of ID2, CD80 and CD86 suggesting antigen presentation functions. Interestingly, the authors described a heterogeneity in unstimulated pDCs with the identification of 9 different clusters driven by the differential expression of 4 genes: *PTGDS*, *TCL1A*, *TCL1B*, and *IGLC2*. The role of these 4 genes in pDCs functions and heterogeneity is not yet understood as there is for now no knowledge of their involvement in pDCs functions. In tumors, it has been demonstrated that pDCs subpopulations are differentially linked to clinical outcome (Sosa Cuevas et al. 2022). For instance, PD-L1- CD80+ pDCs were enriched in tumors of melanoma patients and were associated with poor clinical outcome.

Overall, it seems that the secretion of IFN-I can be attributed to a small fraction of pDCs rather than the whole subset, and the same conclusion can be made for their antigen presentation capacity. Nevertheless, all these results have to be confronted with the work of *Abbas et al.*. After infecting mice with MCMV virus during 33h, 36h and 48h, they sorted pDCs from spleens, then performed sc-RNA sequencing and obtain pDC activation trajectory with pseudo time analysis (Abbas et al. 2020). Their elegant results show that, in mice, pDCs will undergo 3 different activation states. First, they will start to secrete low quantities of IFN-I. Then, their IFN-I production will increase along with their production of IL-12, to finally acquire a fully mature activation state where they will lose their IFN-I production in the favor of the high expression of MHC-II and CCR7. Evidence of this pDCs trajectory has yet to be demonstrated in humans. But their work reminds us the importance of several time points studies and how instead of looking at different pDC subpopulations, we might be looking at pDCs maturing with different kinetics.

## **E. TA-pDCs are associated with an immunosuppressive response**

In the TME, pDCs exhibit a partially activated state and lose their ability to secrete IFN- $\alpha$  as described in breast cancer (Sisirak et al. 2012), ovarian cancer (Labidi-Galy et al. 2012), head and neck cancer (Hartmann et al. 2003), melanoma (Terra et al. 2018), lung (Perrot et al. 2007), and glioma (Dey et al. 2015). Different element present in the TME can cause this inhibition.

### **1. Element leading to the inhibition of IFN-I production by TA-pDCs**

#### **a) Soluble factors inhibiting IFN-I secretion by TA-pDCs**

Our lab previously demonstrated that pDCs exposure to primary dilacerated tumors (SN-Dil) inhibited their secretion of IFN-I (Sisirak et al. 2013). It is the transforming growth factor  $\beta$  (TGF-

$\beta$ ) cytokine, highly present in SN-Dil, that causes this inhibition. The use of TGF- $\beta$  antagonist confirmed the contribution of tumour-derived TGF- $\beta$  in pDC suppression that restored TLR-induced IFN- $\alpha$  production of pDCs (Bekeredjian-Ding et al. 2009). Interestingly, TGF- $\beta$  reduced CCR7 expression but increased CXCR4 expression, resulting in pDCs migration impairment to the tumor-draining lymph node (Bekeredjian-Ding et al. 2009). We also demonstrated that TGF- $\beta$  synergized with TNF- $\alpha$  to inhibit IFN-I production by pDCs. Tumour-derived prostaglandin E2 (PGE2) can also impair pDC production of IFN-I (Bekeredjian-Ding et al. 2009).

### **b) Membrane factors inhibiting IFN-I secretion**

#### **In cancer:**

Surface membrane protein, such as ILT7, can also inhibit IFN-I production. IFN- $\alpha$  secreted by pDCs may induce bone marrow stromal antigen 2 (BST2), known as tetherin or ILT7 Ligand, on cancer cells which will interact with inhibitory receptor expressed by pDCs: immunoglobulin-like cell transcript 7 (ILT7) (Cao et al. 2009). However, the activation of ILT7 inhibit the production IFN-I in pDCs. it was suggested that a negative feed-back loop involving a cross-talk between pDCs and cancer cells is involved in the inhibition of IFN-I secretion (Cao et al. 2009) [Figure 4]. In addition, several human cancer cells overexpress BST2 (Tiwari et al. 2019) explaining the inhibition of IFN-I production by pDCs in the majority of tumors.

#### **In autoimmunity:**

Cutaneous and systemic lupus erythematosus (SLE) are autoimmune diseases that affect multiple organ systems, including the skin, joints, and kidneys. The pathogenesis of SLE is complex but involves dysregulation of the immune system in which pDCs and their production of IFN-I play a crucial role (J.-M. Kim et al. 2015). In SLE, pDCs are activated by immune complexes composed of antibodies interacting with self-nucleic acids (Mustelin, Lood, and Giltaiy 2019), which induced an excessive and prolonged secretion of IFN-I (J.-M. Kim et al. 2015) causing tissue damage (Crow 2014). Hence, targeting pDCs to decrease their

production of IFN-I have been explored as a potential therapeutic strategy for SLE. It is in this context that it was first described that targeting BDCA2 with anti-BDCA2 antibody will engaged their internalization in the endosome, preventing its interaction with immune complex and thus inhibiting the production of IFN-I by pDCs (Pellerin et al. 2015; Blomberg et al. 2003; Furie et al. 2019). Nowadays, new immunotherapies using anti-BDCA2 antibodies are currently in clinical trial in to assess their benefits (Werth et al. 2022).

### **In infection:**

Another surface protein involved in the inhibition of IFN-I production are protein of the Siglec family. Even though Siglec proteins have been associated with antigen uptake and bacteria phagocytosis. In mice its engagement with anti-Siglec-H antibody results in the inhibition of IFN- $\alpha$  production in response to TLR9 stimulation (A. Blasius et al. 2004). Different explanation on the mechanism underlying the production of IFN-I inhibition has been proposed (A. L. Blasius and Colonna 2006) but remains to be tested *in vitro* and *in vivo*.

The involvement of BDCA2 and Siglec proteins in the inhibition of IFN-I in the context of tumor growth have yet to be unravel.

## **2. Mechanisms underlying the inhibition of IFN-I production**

TGF- $\beta$  from breast cancer impaired TLR signaling by decreasing the expression of interferon regulatory factor 7 (IRF7) and inhibiting its nuclear translocation (Sisirak et al. 2013). Furthermore, the work of Hartmann et al. observed a downregulation of TLR9 in TA-pDCs from HNSCC, correlating with the loss of IFN-I inhibition (Hartmann et al. 2003). Indeed, TLR9 location and trafficking can be impacted by TGF- $\beta$ . We demonstrated that TGF- $\beta$  induce a sustain activity of BAD-LAMP which controls TLR9 trafficking (Combes et al. 2017). By sequestering TLR9 in the late endosome, TLR9 cannot activate the production of IFN-I in pDCs any longer.

### **3. Induction of tolerogenic T responses by TA-pDCs**

#### **a) IDO production by pDCs**

In mice, a small fraction of pDCs constitutively express Indoleamine 2,3 DiOxygenase (IDO) has been identified in tumor draining lymph node (tdLN) (Sharma et al. 2007; Munn et al. 2004). IDO is an enzyme that degrades tryptophan into kynurenine, a toxic metabolite (Mellor and Munn 2004). Kynurenine binds aryl hydrocarbon receptor (AhR) and leads to Treg polarization (de Araújo et al. 2017). More than producing toxic metabolites, tryptophan degradation will lead to tryptophan deprivation and inhibit T cell priming, proliferation, and will decrease their effector functions (WanJun Chen 2011). In vitro, tdLN IDO+ pDCs activated resting Tregs from non-tumor-bearing hosts without the need for mitogen or exogenous anti-CD3 activation (Sharma et al. 2007). Adoptive transfer of tdLN DCs into naive mice induced a strong T cell anergy.

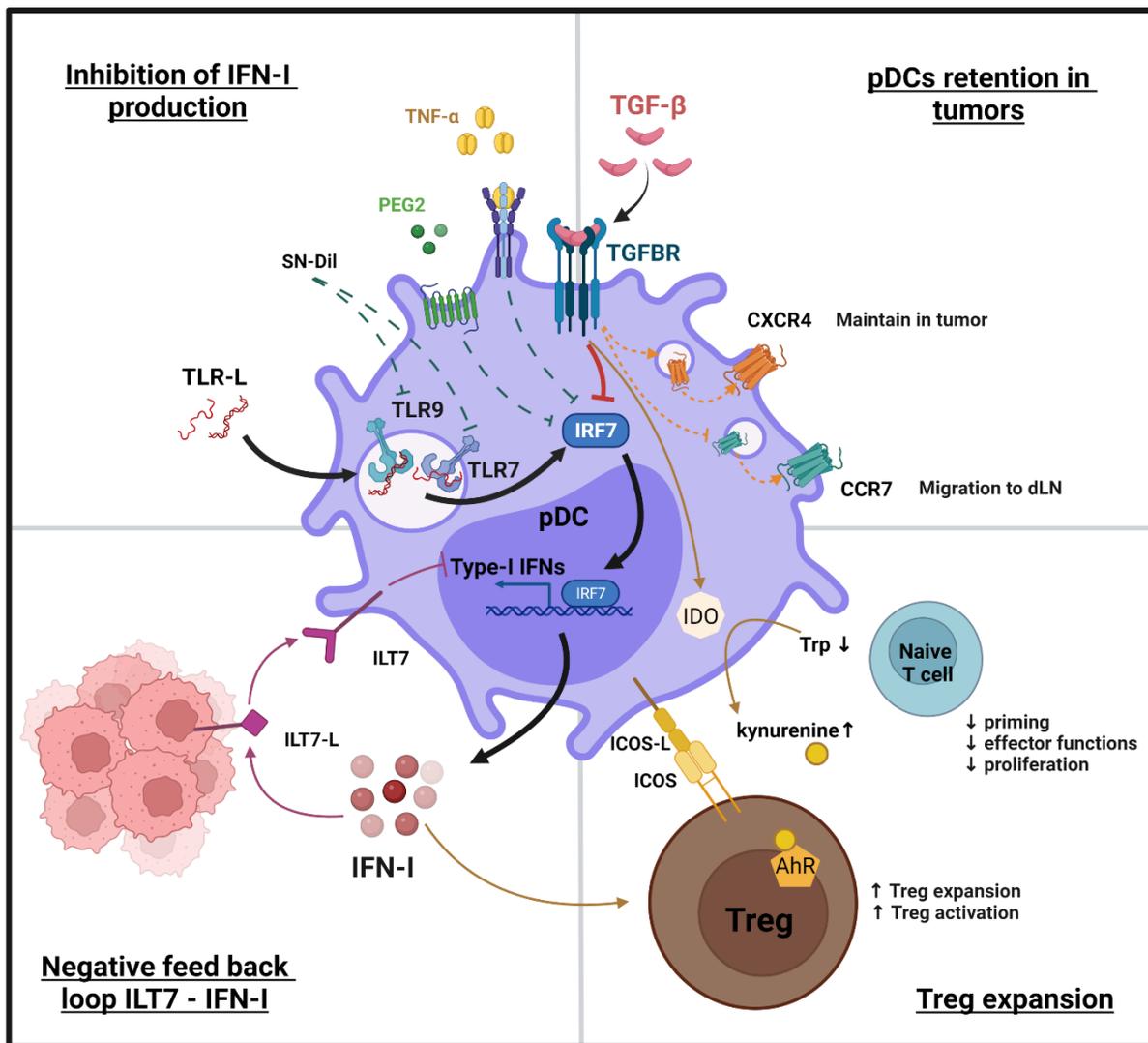
IDO expression by pDCs can be significantly up-regulated when stimulated with TLR agonists (Wei Chen et al. 2008; Manches et al. 2008), but can also be induced upon TGF- $\beta$  stimulation (WanJun Chen 2011). It also has been demonstrated that a positive feedback loop can be put in place in pDCs upon TGF- $\beta$  stimulation. TGF- $\beta$  will induce IDO activation and IDO will activate signaling pathways leading to the production of more TGF- $\beta$  (WanJun Chen 2011). Overall, IDO triggers T cell dysfunction and promotes tumor immune-escape. Finally, a new pDCs subpopulation was described as CD2Hi, CD5+, and CD81+ pDCs and were able to induce a more important Treg differentiation, correlated with a slightly higher production of IDO and reduced IFN-I production compared to CD2Hi CD5- CD81- pDCs (H. Zhang et al. 2017). This new population is present in human peripheral blood and in the bone marrow.

#### **b) pDCs specific interaction with Treg**

In several cancer types, an increase in TA-pDCs correlates with an increase in Tregs (Sisirak et al. 2012; Conrad et al. 2012; Faget et al. 2012) and a decreased overall survival (Treilleux

et al. 2004; Labidi-Galy et al. 2012; Gousias et al. 2013; Aspod et al. 2013). One explanation is that the absence of IFN-I, TA-pDCs favored immunosuppressive T cell responses by inducing Treg expansion (Sisirak et al. 2012). TA-pDCs express ICOS-L and drive the activation and expansion of ICOS+ Foxp3+ Treg cells in breast (Faget et al. 2012), ovarian (Conrad et al. 2012), gastric (Huang et al. 2014), and liver cancers (Pedroza-Gonzalez et al. 2015). ICOS-L+ pDCs interaction with ICOS+ FoxP3+ Tregs likely contribute to the deleterious impact of pDCs on tumor progression. Finally, we showed that it is only in the absence of IFN-I that pDCs can induce T reg expansion through ICOS-L/ICOS interaction.

All together understanding the mechanisms inhibiting IFN-a production by TA-pDCs would help use to restore pDCs functions and could stablish an antitumoral TME.



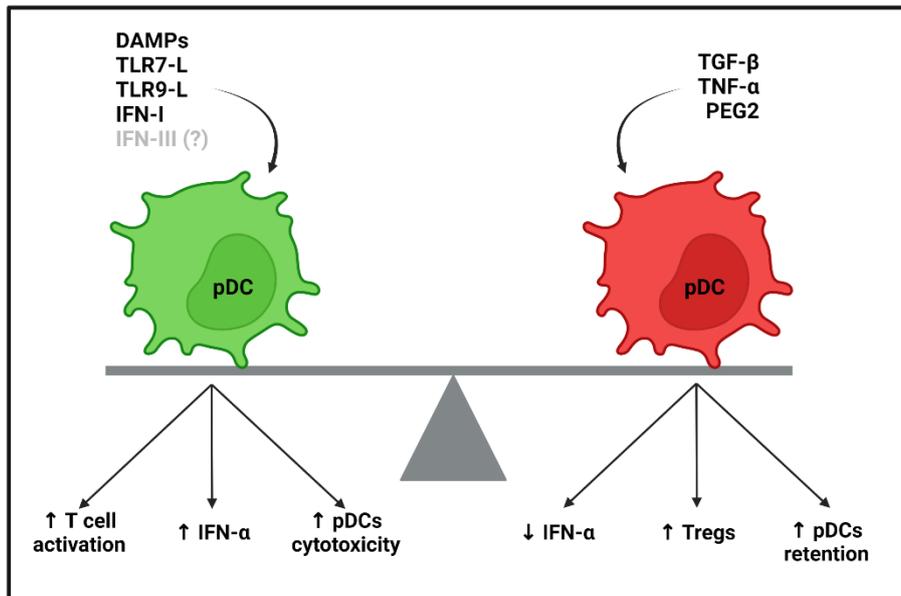
**Figure 4 – TA-pDC tolerogenic functions**

The main activation pathways for pDCs (black arrows) is induced by TLR ligands, such as Imiquimod or CpG which can activate TLR7 or TLR9 respectively. TLR7 or TLR9 activation will lead to the nuclear translocation of IRF7 leading to the production of IFN-I. However, this TLR activation can be inhibited by different elements (left upper square). Indeed, once exposed to SN-Dil pDCs lose their ability to secrete IFN-I. It has been demonstrated that both PEG2 and TNF- $\alpha$  inhibit IFN-I production by pDCs. The main cytokine responsible for the inhibition of IFN-I secretion is TGF- $\beta$  that decrease IRF7 expression, and its translocation to the nucleus. Moreover TGF- $\beta$  inhibit CCR7 but increases CXCR4 expression, which impairs pDCs migration to the tumor draining lymph node where they could activate tumor specific T cells (right upper square). pDCs express high level of ICOS-L in the TME, and ICOS-L is involved in Treg expansion (right lower square). TGF- $\beta$  also increase IDO activation leading to T cell dysfunctions (right lower square). Finally, pDCs own secretion of  $\lambda$ I can be part of a negative feedback loop, as it induces ILT7-L expression on cancer cell. ILT7-L will interact with its receptor ILT7 at the surface of pDCs leading to the inhibition of IFN-I (left lower square).

## **F. TA-pDCs Dual Role: A balance within the TME**

Hot tumors are defined by their capacity to induce a strong anti-tumor immune response. Important characteristics of hot tumor are: their high infiltration in CD3+ and CD8+ T cells and the presence of pro-inflammatory cytokines in the TME. On the contrary, cold tumors can be characterized as immunosuppressive tumors and have a poor response to immune checkpoint inhibitors (ICI) such as anti-PD-L1 and anti-CTLA-4 antibody (J. M. Kim and Chen 2016; Hegde, Karanikas, and Evers 2016). As hot tumors respond better to ICI, strong effort were directed toward the possibility to turn cold tumors into hot tumors (Y.-T. Liu and Sun 2021). Different elements present in the TME could severely impact pDC functions as mentioned before. PAMPs such as glycoproteins present at the surface of cancer cells or apoptotic cancer cells can trigger secretion of IFN-I by pDCs through TLR7 or TLR9 activation. But it could also trigger antigen uptake and processing by pDCs that will then induce T cell activation. Nevertheless, there are strong inhibitory signals in the TME such as TGF- $\beta$  that can abrogate all pDC functions. In the continuity of turning cold into hot tumors, different ways to reactivate pDCs, but above all to re-establish IFN-I production, are currently under study. Even though it has been well demonstrated that pDCs activation with TLR7 and TLR9 is directly linked to a strong IFN-I production *in vitro* (Di Domizio et al. 2009; Saitoh et al. 2017), the same demonstration with human TA-pDCs *in vivo* has yet to be done. The same is true for IFN-I pDCs stimulation. *In vitro*, we know that such stimulation induces an IFN-I production positive feedback loop (Y.-J. Liu 2005), but no evidence supports this observation *in vivo* and in the context of cancer. Here we propose the hypothesis were pDC activation state depends on a balance between immunostimulatory or immunosuppressive cytokines present in the TME [Figure 6].

Controlling the presence and the action of these cytokines with inhibitors or by adding exogenous TLR-ligands and cytokines could restore pDC immunostimulatory functions.



**Figure 6 – pDC dual role in TME**

TA-pDCs dual role in tumors could be highly dependent on element present in tumors, which could have a stronger impact on their functions and overcome their intrinsic functions. DAMPs present in the TME can activate pDC immunostimulatory functions such as T cell activation and IFN-I production. However, this activation can be inhibited by immunosuppressive cytokines, such as TGF- $\beta$ , TNF- $\alpha$ . PEG2 can also inhibit pDC activation. A way to restore TA-pDCs is to add TLR7 or TLR9 ligands which have are involved *in vitro* in increase T cell activation, IFN-I production and pDCs cytotoxicity. IFN-I cancer treatment could also increase IFN-I production by pDCs as described *in vitro*. The main challenge is now to asses if immunosuppressive TA-pDCs (in red) can be reprogrammed to become immunostimulatory TA-pDCs (in green) thanks to immunotherapies aiming to create a TME in favor of tumor regression.

## **G. pDC-based Cancer immunotherapies**

### **4. Immunotherapy Strategies Based on Activation of TLR Signaling**

Even if ICI validated the use of immunotherapies in cancer treatment, only a small fraction of patients does respond to those treatments. Therefore, we need new immunotherapy strategies. One interesting strategy is the use of TLR agonist that are a great way to elicit a strong immune response. TLRs can be located at the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) but can also be located in endosomes (TLR3, TLR7, TLR8, TLR9). Each immune cell will have a range of TLRs expression [Table 3]. Although cDCs and pDCs are extremely rare in the TME, they play a pivotal role in the anti-tumor immune response as they are at the interface between innate and adaptive immune response. But also because they have the ability to induce the appropriate immune response depending on the TLR that has been activated. As only TLR7 and TLR9 will induce cellular adaptive immune response in pDCs, we will focus on the immunotherapies targeting those TLRs

#### **c) In mice**

Our team demonstrated intratumoral injection of TLR7 agonist in mice mammary tumors restore TApDC function, previously inhibited by the TME, and caused a significant tumor growth delay (Le Mercier et al. 2013). In mice, treatment of basal cell carcinoma and melanoma with IMQ, induce tumor regression in a pDC-dependent manner (Palamara et al. 2004). CpG injection within mice bearing melanoma tumor favored the tumor clearance (C. Liu et al. 2008). In this study they showed, that CpG activated pDC had a strong secretion of inflammatory chemokines such as CCL3, CCL4 and CCL5. These chemokines attracted NK cells within the TME. Moreover, CpG induced IFN-I production and OX40L expression by pDCs. These two mechanisms were directly link to NK cell cytotoxicity and IFN- $\gamma$  secretion respectively. In this quite complet study they also demonstrated that CpG activated pDCs were associated with CD8+ T cell increased activity on tumor cell destruction (C. Liu et al. 2008). More studies

successfully demonstrated CpG activated pDCs ability to active CD8+ T cell (Salio et al. 2003; Lou et al. 2007).

Nevertheless, these observations did not help to characterize the mechanism involved in CD8+ T cell activation by pDC, whether it is direct via antigen presentation, or indirect through other immune cells. In Lou et al.'s model indicates that CpG activated pDC may have antigen presenting capacities *in vivo* and elicit CD8+ T cells antitumor response via antigen seem to be as efficient as cDC to elicit anti-tumor CD8 T cell (Lou et al. 2007)

#### **d) In humans**

As triggering TLR9 could boost TA-pDC functions, cancer immunotherapies involving TLR9 were extensively studied as reviewed by *Pahlavanneshan et al.* For instance, CpG-7909 was tested in renal cell carcinoma (RCC), glioblastoma, cutaneous T cell lymphoma, NSCLC, non-Hodgkin's lymphoma, and melanoma. Overall, TLR9 agonists are promising immunotherapy, but are unfortunately associated with severe adverse events as Cytokine Release Syndrome (CRS). Other adverse event has been reported such as injection site reaction following subcutaneous and intra-tumoral injection. As TLR9 induced severe adverse events, other strategies with lesser adverse events should be investigated.

Concerning the use of TLR7 agonist, Imiquimod was tested to treat human papillomavirus (HPV)-induced warts, instead of surgical resection. IMQ treatment was well tolerated and showed encouraging results (C. J. de Witte et al. 2015). Flu-like symptoms, myalgia, or lymphadenopathy systemic effect were not reported in any patients. However, only 21 patients were included in the clinical trial and information lacked uniformity in the defined endpoints. Moreover, The US Food and Drug Administration (FDA) approved the use of IMQ for superficial basal cell carcinoma (BCC) treatment. Indeed, after 6 weeks of treatment with IMQ a complete regression of BCC was observed for the seven patients enrolled in this study (Stary et al. 2007). IMQ treatment of BCC increased pDCs infiltration in the TME, and ~30% of the TA-pDCs produced IFN- $\alpha$ . Even more interesting, BCC islets stained positive for TRAIL-R, and are surrounded by TRAIL+ pDCs suggesting that BCC IMQ treatment induced direct cytotoxic

properties in TA-pDCs (Stary et al. 2007). In the light of pDCs potential heterogeneity, it would have been interesting in having a double staining against IFN- $\alpha$  and TRAIL to observe if there are double positive IFN- $\alpha$ + TRAIL+ TA-pDCs or if there are two very distinct populations IFN- $\alpha$ + pDCs *versus* TRAIL+ pDCs. A phase two clinical trial is currently evaluating the combination of IMQ with chemotherapy (paclitaxel) to treat cutaneous breast cancer metastases (Salazar et al. 2017). Even if IMQ treatment is approved for BCC treatment, as a cream for dermal application, efforts should now be made to use it for other types of cancers.

<b>[Table 3] TLR expression in immune cells</b>		
<b>TLR</b>	<b>Expression on Immune cell</b>	<b>Level of expression</b>
TLR1	ALL	High or Low
TLR2	cDCs	High
	Monocytes	High
	T cells	High
TLR3	DCs	High
	NK cells	High
	T cells	High
TLR4	cDCs	High
	Macrophages	High
	T cells	High
TLR5	Monocytes	High
	cDCs	High
	NK cells	High
	T cells	High
TLR6	B cells	High
	cDCs	High
	pDCs	Low
	Monocytes	Low
	NK cells	Low
TLR7	B cells	High
	cDCs	High
	pDCs	High
	Monocytes	High
	T cells	High
TLR8	Monocytes	High
	cDCs	High
	NK cells	Low
	T cells	Low
TLR9	pDCs	High
	B cells	High
	Macrophages	High
	NK cells	High
TLR10	B cells	High
	cDCs	Low
	pDCs	Low

## 2. pDC-Based Vaccines for Cancer Immunotherapy

DC-based vaccines consist in expanding moDCs or cDC2 and stimulating them *in vitro* before injection into cancer patients. It is also possible to cultivate moDC or cDC2 with TAA to favor antigen uptake and processing to increase specific antitumor immune response. Unfortunately, this strategy has shown low efficacy probably due to a tolerogenic TME inducing DC dysfunctions, or probably because patients enrolled in the clinical trials were in late stages of disease (Filin et al. 2021). Combining DC-based vaccines with ICI shows promising results, suggesting that further investigation shall be done on the appropriate use of DC-based vaccines (Filin et al. 2021).

Instead of using moDCs or cDC2, pDCs could be used. They can be retrieved from 3 different sources: from peripheral blood, from hematopoietic stem and progenitor (HSPC) cell that will mature into pDCs thanks to the right combination of cytokines (Laustsen et al. 2018), or from a pDC cell line originated from leukemic pDCs (Charles et al. 2020). As reviewed by *S.Hernández et al.* there are currently 3 clinical trials evaluating adoptive transfer of TAA loaded-pDCs. All three clinical trials showed an increased immune response with an increase of tumor specific CD4+ and CD8+ T cells in the tumor bed, and increase of tumor specific T cell activation and production of IFN- $\gamma$  (Hernández, Jakobsen, and Bak 2022). Even if these pDCs-based vaccines induced an appropriate T cell response, all came with light to severe adverse events such as flu-like symptoms up to upper respiratory infections and lymphadenitis. In terms of underlying mechanisms involved in pDC-based vaccine, it has been reported that FSME-activated pDCs will secrete high levels of CXCR3 ligands and thus attract cytotoxic lymphocytes compared to FSME-activated cDC2 (van Beek et al. 2020). These observations were confirmed in skin biopsies of melanoma patients who had a strong infiltration of effector lymphocytes after pDC injections (van Beek et al. 2020). The authors also suggested the use of both cDC2 and pDCs together to combine chemoattractive properties of pDCs with the superior T cell priming capacity of cDC2. This type of vaccine has already been tested in

prostate cancer patients (NCT02692976) and is being evaluated in metastatic endometrial cancer (NCT04212377).

Because it has been demonstrated that pDCs have 3 main immunostimulatory functions, antigen presentation, direct cytotoxicity, and IFN-I production, pDCs represent an interesting target for new immunotherapies. Nevertheless, these antitumor functions can be repressed in the TME due to the presence of TGF- $\beta$ , TNF- $\alpha$ , and PEG2. A strategy to reactivate pDCs is to use TLR7 or TLR9 agonists. However, the use of TLR9 agonists has been associated to severe adverse events such as CRS, where TLR7 agonists were rarely associated with moderate adverse events. Thus, IMQ was validated by the FDA as dermal application for BCC treatments. But there is a possibility that if IMQ was injected into the tumor a strong adverse event as TLR9 agonists could be present. Other than TLR triggering or DC based vaccines, finding a new strategy to reactivate TA-pDCs, as they could be key to restore an immunostimulatory TME leading to tumor regression, with the fewer and weaker adverse event as possible, could be an important breakthrough in immunotherapies.



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## **CHAPTER II**

### *Similarities and differences between IFN-I and IFN-III in the anti-tumoral immune response*

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#### **IV. IFN description**

##### **A. IFN families**

Interferons (IFNs) are a group of cytokines that play a crucial role in the antiviral and antitumor immune responses. There are three main classes of IFNs with specific functions, Type I Interferons (IFN-I), Type II Interferons (IFN-II), and Type III Interferons (IFN-III). These three IFN families can be distinguished from one another by their genomic locus, similarities in the DNA sequences, and their share of the same signaling receptors [Table 4].

##### **6. The large IFN-I family**

Nowadays, there are 17 types of IFN-I cytokines in humans. The first discovered cytokines were IFN- $\alpha$ , with its 13 subtypes (IFN- $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 6,  $\alpha$ 7,  $\alpha$ 8,  $\alpha$ 10,  $\alpha$ 13,  $\alpha$ 14,  $\alpha$ 16,  $\alpha$ 17, and  $\alpha$ 21), and IFN- $\beta$ . In 1985 IFN- $\omega$  was discovered and linked to the IFN-I family (Capon, Shepard, and Goeddel 1985). It is only sixteen years later, in 2001, that IFN- $\kappa$  will be discovered and described as an IFN-I (LaFleur et al. 2001). The newest member on this family is IFN- $\epsilon$  described in 2004, that also shares genomic locus and functional receptors with rest of the IFN-I family, and have sequence similarities with IFN- $\alpha$  and IFN- $\beta$ . An elegant paper by *Hertzog et al.* review the similarities of these 17 different cytokines (Hertzog et al. 2016). In the context of viral infections, IFN-I can be produced by infected epithelial cells, or by immune cells after activation. IFN-I stimulated cells produce antiviral proteins and inhibit viral replication. In the cancer, IFN- $\alpha$ 2 has been approved by the FDA to treat certain severe cancers such as leukemia, myeloma, and lymphoma, as it is a key cytokine involved in antitumor immune response, but also in direct tumor regression.

## **7. IFN-II and its unique cytokines**

IFN-II contains only one cytokine which is IFN- $\gamma$ . IFN- $\gamma$  is located on the chromosome 12, which differentiated it with IFN-I, as they are located on the chromosome 9, and binds a tetramer formed of two IFNGR1 chains and two IFNGR2 chains. T cells and NK cells are the main producers of IFN- $\gamma$  which play a primary role in cell-mediated immunity. The role of IFN- $\gamma$  in the TME is still controversial, as it has been described to elicit antitumor as well as pro-tumorigenic immune responses (Jorgovanovic et al. 2020). It can activate perforin and granzyme B secretion to induce cancer cell apoptosis (Tau et al. 2001; Maimela, Liu, and Zhang 2019), but can also increase PD-1 and CTLA-4 expression along with IDO, three proteins associated with immune-suppressive mechanisms (Mojic, Takeda, and Hayakawa 2017; Zaidi et al. 2011).

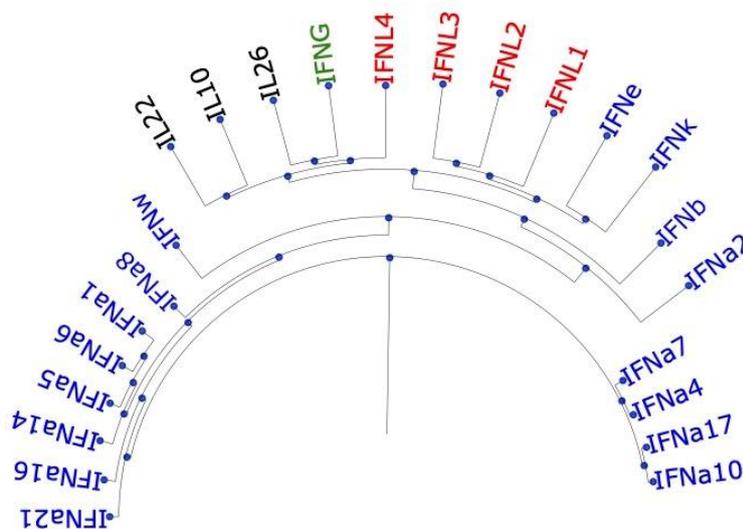
## **8. IFN-III, the most recently discovered family**

The IFN-III family was first described in 2002 (Kotenko et al. 2002; Sheppard et al. 2002). In humans, the IFN-III family is composed of IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3 or IL-29, IL-28A and IL-28B respectively and are located on the chromosome 19. A fourth protein belonging to the IFN-III family has been described eleven years later, IFN- $\lambda$ 4 (Ludmila et al. 2013). Although IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3 have a strong homology between them, IFN- $\lambda$ 4 only shares 30% homology with them and its expression depends on the presence of a polymorphism located in the first exon of its gene sequence (O'Brien, Prokunina-Olsson, and Donnelly 2014). When mentioning homology, IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3 have a strong homology between them but share only 10 to 15% of homology with IFN-I, and rather have a structure similar to the IL-10 cytokine family (Gad et al. 2009).

IFN-III first interacted with the IL-28R $\alpha$  chains (Sheppard et al. 2002), then recruited the IL-10R $\beta$  chain (Yoon et al. 2010) to form the heterodimer IFN-Lambda Receptor (IFNLR). Nevertheless, IFN-III and IFN-I have relatively similar functions. In mice, IFN- $\lambda$ 1 is a pseudogene, only IFN- $\lambda$ 2 and IFN- $\lambda$ 3 can be found in protein (Lasfar et al. 2006), and no evidence for an IFN- $\lambda$ 4 coding gene was found in mice.

This group of IFNs is involved in immune response against viral infection, particularly in mucosal immune cells and triggers the production of antiviral proteins as IFN-I family do. Recently, numerous studies evaluated the role of IFN-III against SARS-Cov2 infections. In SARS-Cov2 infection IFN-I and IFN-III does not seem to be produced, however pre-treatment with these cytokines reduce virus replication, with IFN-III being more potent (Y.-M. Kim and Shin 2021). IFN-III also seems to have antitumor properties as we will discuss later on.

In summary, each IFNs families have their own genomic locus and receptors, activating a specific range of epithelial and immune cells, leading to potential distinct roles in antitumor immune responses.



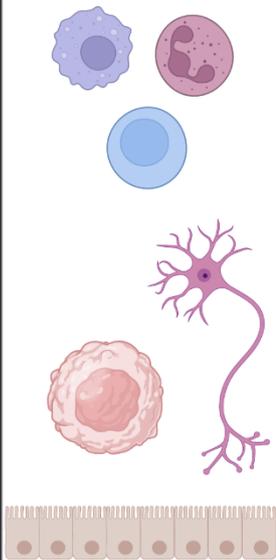
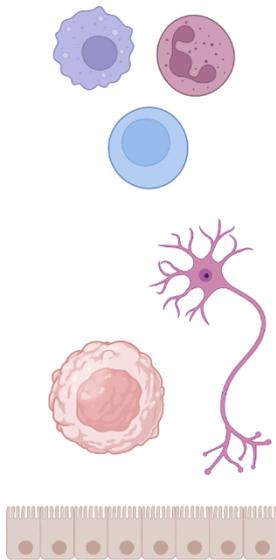
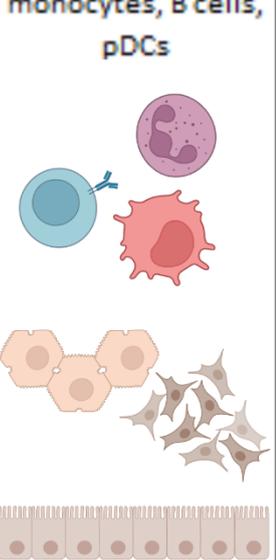
**Figure 7 – Phylogenetic tree of IFNs Families**

Amino acid sequence of 24 different cytokines were retrieved from Uniprot website and aligned with Bio Python packages. The phylogenetic tree based on amino acid and secondary structures variation was constructed with Bio Python packages. IL-22, IL-10, IL-26, three cytokines belonging to the IL-10 family (in black) seems to originate from IFN-I family (in blue) and are closely related to IFN- $\gamma$  (in green) in term of amino acid sequence and secondary structure. IFN- $\lambda$ 4 is also closely related to IL-10 and IFN-II family before being linked to the other members of IFN-III family (in red), IFN- $\lambda$ 1, IFN- $\lambda$ 2, and IFN- $\lambda$ 3. Finally, IFN- $\lambda$ 1, IFN- $\lambda$ 2, and IFN- $\lambda$ 3 have amino acid sequence or secondary structure similar to IFN- $\kappa$  and IFN- $\epsilon$ , the most recent members of IFN-I family. IL-10, IFN-II and IFN-III seem to derive from IFN-I with IFN- $\beta$  and IFN- $\alpha$ 2 being the closest to the other IFN families in terms of protein modification.

## 9. Expression profile of IFNLR on immune cells

On the contrary of IFNAR1 and IFNAR2 that are ubiquitously express throughout human cells, IL-28R $\alpha$  expression is restricted to certain type of epithelial cells and immune cells. For epithelial cells, IL28-R $\alpha$  is expressed in the digestif track (Brand, Beigel, et al. 2005; Kotenko et al. 2002; Sheppard et al. 2002), in the respiratory track (Kotenko et al. 2002; Sheppard et al. 2002; Sommereyns et al. 2008), in kidney (Kotenko et al. 2002; Sheppard et al. 2002; Sommereyns et al. 2008), but also in keratinocyte (Maher et al. 2008; K. Witte et al. 2009). IL28-R $\alpha$  is also expressed by hepatocytes (Doyle et al. 2006; Sommereyns et al. 2008), and it has been shown that IL-28R $\alpha$  mRNA level can be increased upon IFN-I stimulation (Duong et al. 2014). In immune cells IL-28R $\alpha$  was described on, monocytes (Yin et al. 2012; Kelly et al. 2016), B cells (K. Witte et al. 2009; Groen et al. 2015; Kelly et al. 2016) and plasmacytoid dendritic cells (pDCs) (Yin et al. 2012; Kelly et al. 2016). IL-28R $\alpha$  is not express on neutrophils at steady state, but can be induced via TLR4 activation (Broggi et al. 2017). Although IL28-R $\alpha$  expression was detected on these different cell types, these analyses were mainly performed by RT-PCR quantifying only mRNA expression of *IFNLR1* gene. But also by western blot, flow cytometry where anti-IL-28R $\alpha$  antibodies are not refined enough to be reliable. For instance, the use of such antibodies lead to the misinterpretation that T cells expressed IL-28R $\alpha$ , which we now know that it is incorrect (Dai et al. 2009; Jordan et al. 2007; K. Witte et al. 2009). Moreover, the presence of IL-28R $\alpha$  at the cell surface is not a sufficient evidence to conclude on cell capacity to respond to IFN-III. Functional study shall be performed in each cell type to asses rather they actually undergo functional modification after IFN-III stimulation. Such experiments were intensively performed in epithelial cells, where it has been demonstrated that IL-29 induces a type 1 Interferon-like program to promote antiviral responses in human hepatocytes (Doyle et al. 2006). It was also shown that neutrophils respond to IFN-III and inhibits some of their functions (Broggi et al. 2017). Finally, IFN-III activate some pDCs immunostimulatory properties (Kelly et al. 2016).

[Table 4] Summary of IFNs and their receptors expression profiles

IFN Family	IFN-I	IFN-II	IFN-III
Cytokines	IFN- $\alpha$ 1, IFN- $\alpha$ 2, IFN- $\alpha$ 4, IFN- $\alpha$ 5, IFN- $\alpha$ 6, IFN- $\alpha$ 7, IFN- $\alpha$ 8, IFN- $\alpha$ 10, IFN- $\alpha$ 13, IFN- $\alpha$ 14, IFN- $\alpha$ 16, IFN- $\alpha$ 17, IFN- $\alpha$ 21, IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\omega$ , IFN- $\kappa$	IFN- $\gamma$	IFN- $\lambda$ 1 IFN- $\lambda$ 2 IFN- $\lambda$ 3 IFN- $\lambda$ 4
Receptor chains	IFNAR1 & IFNAR2	IFNGR1 & IFNGR2	IL-28R $\alpha$ & IL-10R $\beta$
Expression profile	Ubiquitously express 	Ubiquitously express 	Respiratory & Digestive track, hepatocytes, renal cells, keratinocytes  Neutrophils, monocytes, B cells, pDCs 

## **B. IFN-III production**

### **10. PRR and IFN-III producing cells**

A broad range of cell can produce IFN-I upon PRR engagement with Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs), and the same is true for the production of IFN-III (Iversen et al. 2010; Levy, Marié, and Durbin 2011). TLR3 activation can induce IFN-III production by cDC1 (Lauterbach et al. 2010; Yoshio et al. 2013; Balan et al. 2014; Murata et al. 2014), by hepatocytes (H.-C. Lee et al. 2014), by epithelial cell from the intestinal and respiratory tract (Ioannidis et al. 2013; Swider et al. 2014), and by modified fibroblast (Casrouge et al. 2006). TLR4 can induce IFN-III production by moDC and macrophages (Coccia et al. 2004; Sirén et al. 2005) at a lesser extent compared to cDC1, but IFN-I treatment will boost TLR4 and TLR3 expression on moDC and macrophages leading to a feedback positive loop of IFN-I and increasing IFN-III production by those immune cells (Sirén et al. 2005). pDCs secrete high amount of IFN-I, but they can also secrete a low dose of IFN-III upon TLR7 and TLR9 stimulation (Coccia et al. 2004; Megjugorac, Gallagher, and Gallagher 2009; Yin et al. 2012; Murata et al. 2014). Once again pDCs stimulation with IFN-I amplifies their production of IFN-III highlighting once again positive feedback loop of IFN-I and IFN-III increase (Yin et al. 2012). If upon infection different cells can produce IFN-III, our lab showed that cDC1 are the cell expressing the highest quantity of IFN-III, and that in the breast cancer TME, cDC1 were the only source of IFN-III, with ~20% of IFN- $\lambda$ 1+ cDC1 (Hubert et al. 2020).

When RIG-I, another PRR belonging to the family of Rig-I-Like Receptor (RLR), is triggered it will recruit the protein MAVS (Mitochondrial antiviral signaling protein) and induce IFN-III production if MAVS was mainly located at the peroxisome membrane. If MAVS is located at the mitochondrial membrane, its triggering will induce IFN-I production (Odendall et al. 2014). Where all the previous PRR induced IFN-III with IFN-I, only Ku70 cytosolic sensor induce the

specific production of IFN-III in HEK293 and THP-1 cells (X. Zhang et al. 2011), with STING as an essential mediator to the signaling pathway (Sui et al. 2017).

## **11. Signaling pathways involved in IFN-III production**

In humans, IFN- $\beta$  production requires the assembly of a transcriptome enhancer complex, where IRF7, IRF3 and NF- $\kappa$ B bind *IFNB* gene's promotor with the help of the high mobility group protein HMG I(Y) to induce IFN- $\beta$  transcription (Thanos and Maniatis 1995). It was demonstrated that IRF7, IRF3 and NF- $\kappa$ B are involved in IFN-III transcription (H.-C. Lee et al. 2014). However, IFN-III transcription does not need the formation of the transcriptome enhancer complex as IFN- $\beta$  does. NF- $\kappa$ B can induce IFN-III transcription independently of IRF3 or IRF7 (Thomson et al. 2009). The fact that IFN-III can be transcribed through IRFs or NF- $\kappa$ B increases its chance to still be produce in pathological context. Indeed, these 2 pathways would have to be inhibited in order to stop IFN-III production, whereas inhibiting only one of these pathways would be enough to inhibit IFN-I production. This is a great advantage in case of viral infection or tumor development which tend to set mechanisms to inhibit IFN-I or IFN-III production.

The inhibition of IFN-III production is quite similar to IFN-I. As for IFN-I inhibition, B-Lymphocyte-induced maturation protein 1 (BLIMP-1) can inhibit IFN-III production because of the presence of a fixation site in IFN-III promotor (Siegel, Eskdale, and Gallagher 2011; Swider et al. 2014). NF- $\kappa$ B subunit p50 is also involved in the inhibition of IFN-III production (Siegel, Eskdale, and Gallagher 2011). Moreover, peptidyl-propyl cis-trans isomerase NIMA-interacting (Pin-1) will induce ubiquitination of phosphorylated IRF3 leading to its degradation by the proteasome (Osterlund et al. 2007). IRF8 is a competitive inhibitor of IRF3 and IRF7 as it will bind IFN-III promotor at the same site decreasing IFN-III production (Osterlund et al. 2007). Finally, Ubiquitin-specific peptidase 22 (USP22) regulating transcription via the control of histone ubiquitination, inhibits ISGs transcription after SARS-Cov-2 infection in order to tightly control IFN-III production.

## C. Signaling pathways induced by IFN-I and IFN-III

### 12. JAK-STAT signaling pathway

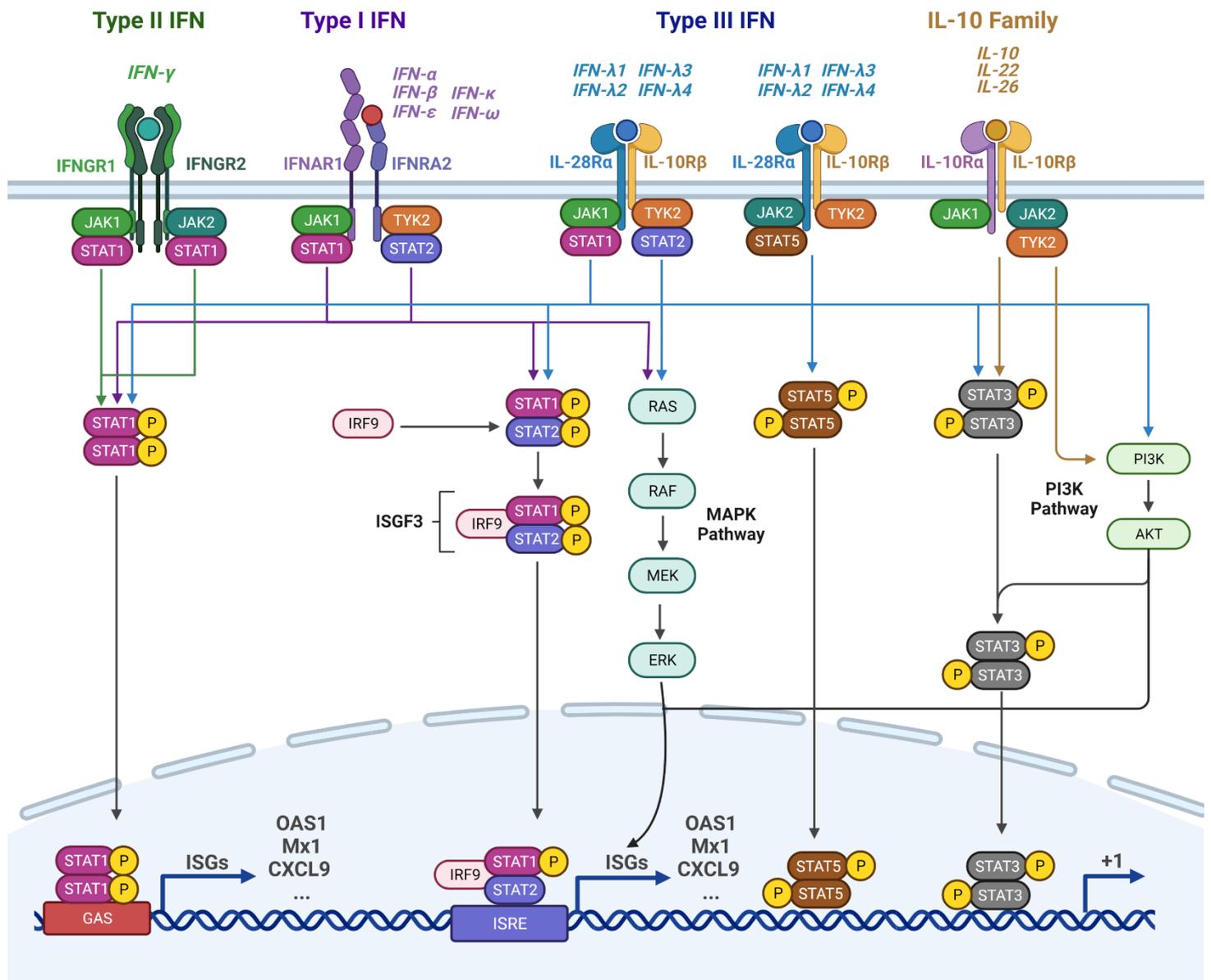
Before to discuss further IFN-III impact on epithelial cells and immune cells, it is important to define the downstream signaling pathways after IFNLR engagement. The canonical signaling pathways following IFN-III stimulation is JAK-STAT signaling as IL-28R $\alpha$  and IL-10R $\beta$  are constitutively associated with JAK1 and Tyk2 kinases respectively (Finbloom and Winestock 1995) [Figure 8]. Upon interaction with the appropriate cytokine, IFNLR will undergo conformational changes creating a ternary complex that is stable enough to facilitate transphosphorylation between Jak1 and Tyk2 leading to their activation (C. Thomas et al. 2011). Both Jak1 and Tyk2 are tyrosine kinase and belong to the Janus kinases (JAKs) family. Hence after the are being activated, Jak1 and Tyk2 will phosphorylate STAT1 and STAT2. Once phosphorylated STAT1 and STAT2 will recruit IRF9 and form the ISGF3 complex. ISGF3 complex will then translocate to the nucleus and bind interferon stimulated response element (ISRE) inducing interferon stimulated gene (ISG) such as OAS1, Mx1, and CXCL9. When STAT1 is inhibited, a complete loss of IFN-III antiviral functions occurs after viral infection (Leiliang Zhang et al. 2011; Pervolaraki et al. 2017). These results highlight the importance of JAK-STAT pathway to induce ISG. There is also evidence that JAK2 can be associated with the IL-28R $\alpha$  chain (Odendall and Kagan 2015) and that STAT3 and STAT5 can be phosphorylation upon IFNLR engagement (Kelly et al. 2016). As IL-28R $\alpha$  interact with IL-10R $\beta$  chains with the IL-10 family, we also looked at IL-10 signaling pathway. IL-10 signaling pathways induced STAT3 phosphorylation (Hutchins, Diez, and Miranda-Saavedra 2013), which could explain why IFN-III can also induced STAT3 phosphorylation when IFN-I and IFN-II do not.

### **13. PI3K and MAPK signaling pathways**

Different evidence demonstrated the involvement the phosphoinositide 3 Kinase (PI3K) pathways and Mitogen-associated protein kinase (MAPK) pathways in IFN-III downstream signaling **[Figure 8]**. Protein belonging to PI3K pathway such as AKT and p38 are phosphorylated in IFN-III stimulated Raji cells (Zhang Zhou et al. 2007). It was also described that PI3K-AKT-GSK3 is involved in the regulation of IL-10 stimulated genes (Antoniv and Ivashkiv 2011). This demonstration reinforces the intertwining between IL-10 and IFN-III that can be explain by the IL-10R $\beta$  common chain. Similarly to IFN-I, IFN-III will also activate MAPK downstream signaling in human fibroblast (Alase et al. 2015) and intestinal epithelial cells (Brand, Beigel, et al. 2005). In the epithelial colorectal cell line HT-29, IFN-III stimulation leads to MEK-1 (Meiosis-specific serine/threonine protein kinase) phosphorylation, followed by ERK1 and ERK2 (Extracellular Signal-regulated kinase) phosphorylation (Pervolaraki et al. 2017).

### **14. Inhibition of IFN-III signaling pathway**

In parallel of ISG induction, SOCS (Suppressor Of Cytokines Signaling) protein such as SOCS1 are also induce by IFN-III as a negative feedback loop inhibiting IFN-III signal transduction (Blumer et al. 2017; B. Liu et al. 2015). It has been shown that when IFN-III signaling is induced SOCS1 inhibition to regulates its own impact of cells. The same mechanism has been described with IFN-I signaling. However, SCOS1 induction may come earlier with IFN-III activation compared with IFN-I, but it will be sustained longer than IFN-I activation. It was suggested that this phenomenon allows a delayed action of ISG induce by IFN-III, but would last longer that IFN-I activation which is very acute (B. Liu et al. 2015). A second mechanism that can inhibit IFN-III signaling, is the neutralization of IFN-III cytokine by a soluble form of IL-28R $\alpha$  chain (K. Witte et al. 2009).



**Figure 8 – Interferon signaling pathways**

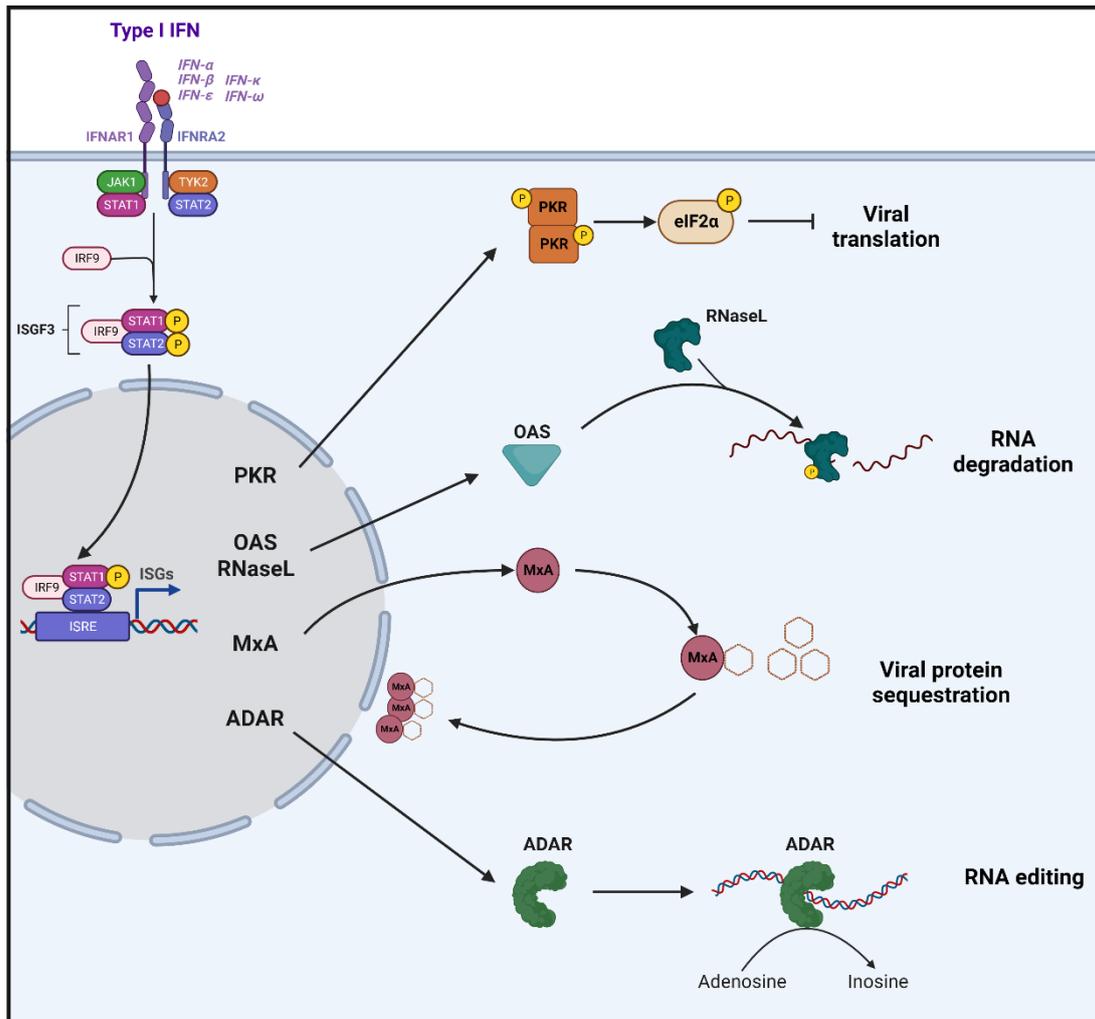
Following IFN-III stimulation IFNLR will undergo conformational and induce Jak1 and Tyk2 transphosphorylation leading to STAT1 and STAT2 phosphorylation. Once phosphorylated STAT1 and STAT2 will recruit IRF9 and form the ISGF3 complex and translocate to the nucleus. ISGF3 will ISRE inducing ISG such as OAS1, Mx1, and CXCL9 transcription. STAT3 and STAT5 can also be phosphorylation upon IFNLR engagement as IL-10 receptor can do too. MAPK pathways is essential for IFN-III downstream signaling and can also be activated by IL-10. Similarly, to IFN-I, IFN-III will also activate MAPK downstream signaling.

## V. Antiviral effect of IFNs

### A. IFN-I and antiviral responses

#### 15. IFN-I induce antiviral responses in epithelial cells

As mentioned before, IFN-I have a pleiotropic effect and will activate ISG transcription through JAK/STAT pathways in epithelial cells. To impair viral replication ISG proteins will inhibit viral protein synthesis, induce degradation of viral RNA, RNA editing, and will sequester viral nucleocapsid [Figure 9]. One of the most studied ISG is double-stranded RNA-activated protein kinase (PKR) (Meurs et al. 1990) that will activate eIF2 $\alpha$  protein, leading to the inhibition of mRNA translation. Two ISG involved in viral RNA degradation are OAS proteins (Ghosh et al. 1991) and RNaseL (A. Zhou, Hassel, and Silverman 1993). It was demonstrated in the context of bunyavirus infection that one role of human MxA protein is to sequester nucleocapsids. MxA will bind nucleocapsids and delocalized around perinuclear area. This mechanism traps viral components and impairs virion formation (Kochs et al. 2002). Another antiviral mechanism is viral mRNA editing by RNA-specific adenosine deaminase (ADAR). ADAR is also an ISG (J. B. Patterson et al. 1995) and binds double-stranded DNA to remove an amino groups (NH<sub>2</sub>) from an adenosine and creates an inosine complex instead (Samuel 2019). This DNA modification impairs viral DNA transcription and translation.



**Figure 9 – Antiviral mechanism induced by IFN-I in epithelial cells**

Following IFN-I stimulation IFNAR will phosphorylate STAT1 and STAT2 and form the ISGF3 complex. ISGF3 translocates to the nucleus to induce ISGs. ISG such as PKR will activate eIF2α to inhibit viral protein translation. Two other ISGs involved in antiviral pathways are OAS, and RNaseL that will degrade viral mRNA. MxA will bind viral nucleocapsid and relocate near peripheral nuclear area impairing virion formation. Finally, ADAR another ISG, transforms adenosine present double-stranded DNA molecule into inosine inhibiting transcription and translation. This mechanism is called RNA editing.

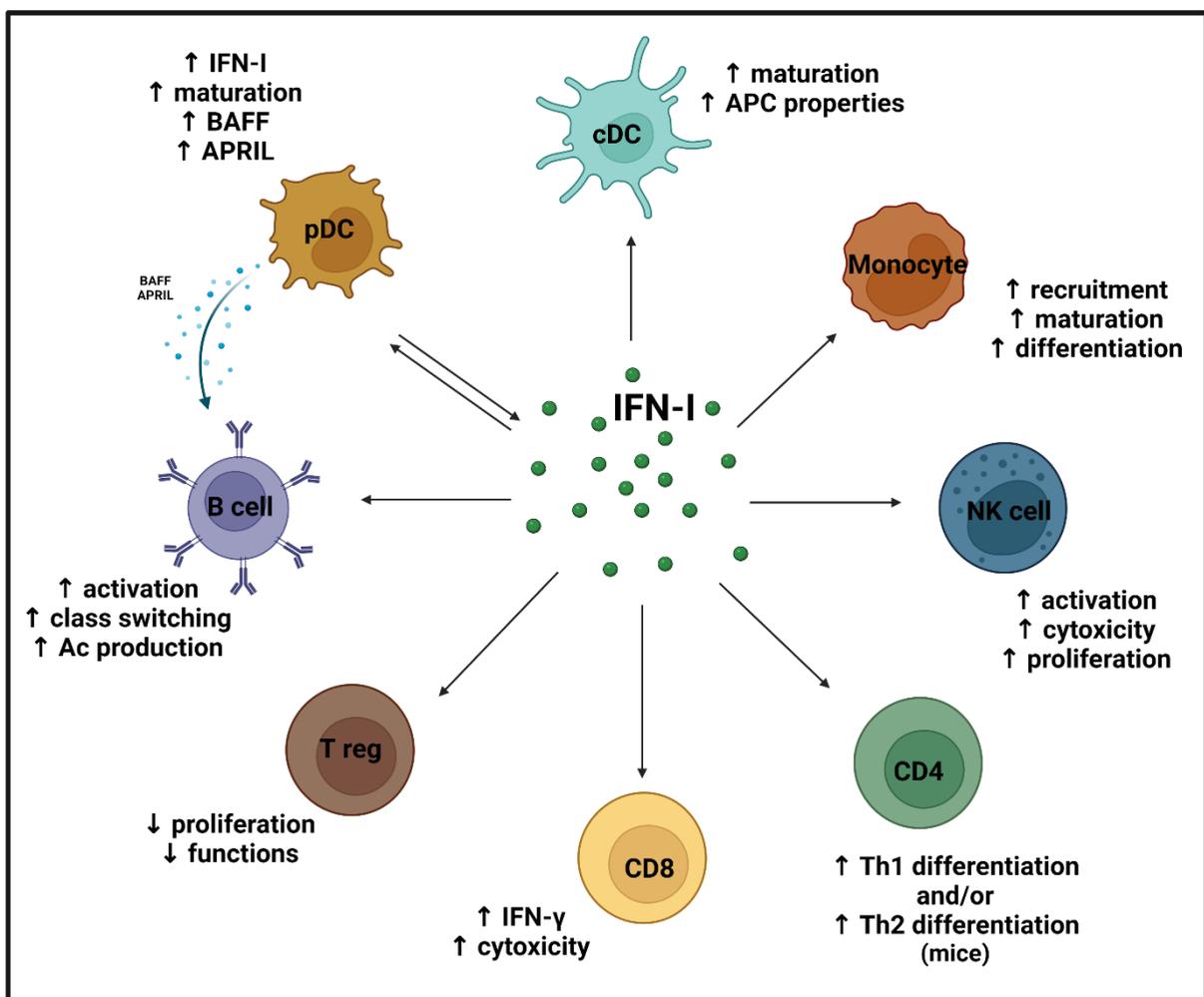
## 16. IFN-I stimulate immune cells to control viral infection

During the early phases of infection, IFN-I will not only act on epithelial cells to limit viral replication, but it will also boost cells from the innate immune system **[Figure 10]**. IFN-I have a strong activation capacity of APC such as monocyte and DCs. It has been described that IFN-I increase monocyte recruitment to infected tissues and increase their maturation (P. Y. Lee et al. 2009). IFN-I also enhance their production of CXCL10 and differentiation into monoDC (Gabriele et al. 2004). On DCs, IFN-I will increase their maturation, their antigen uptake and processing (Simmons et al. 2012).

On pDCs, IFN-I can amplify their own production of IFN-I (Yin et al. 2012). IFN-I enhance NK cell activation, cytotoxicity, and is involved in their proliferation (Swann et al. 2007). All together, these results demonstrate the importance of IFN-I secretion and action on innate immune cells to boost APC, and thus enhance the following adaptive immune response. IFN-I will not only have an indirect effect on adaptive immune response, but also a direct effect as it can impact T cells and B cells **[Figure 10]**. On CD4<sup>+</sup> T cells the role of IFN-I is still controversial in viral infection, different and opposing roles of type I IFNs in the priming and polarization of CD4<sup>+</sup> T has been reviewed (Kuka, De Giovanni, and Iannacone 2019). In mice, IFN-I can increase Tbet1 expression, an important transcription factor for Th1 polarization. However, those results are counterbalanced by observations of Bcl-6 and CXCR5 enhancement in CD4<sup>+</sup> T cells leading to differentiation into T follicular helper cell (Tfh). These observations seem to be caused by different experimental settings and/or different pathogen activations. IFN-I direct effect on human CD8<sup>+</sup> T cells is less controversial as there are clear evidence of IFN-I enhancing IFN- $\gamma$  production along with their cytotoxicity (Curtsinger, Johnson, and Mescher 2003; Curtsinger et al. 2005; Mescher et al. 2006). IFN-I also directly attenuate Treg functions (Gangaplara et al. 2018). In mice IFN-I also negatively impact Treg function and proliferation in mice (Srivastava et al. 2014) and in humans (Piconese et al. 2015). Finally, IFN-I have an indirect effect on B cells as it stimulated pDCs to secrete a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF) (Ding et al. 2009). Kiefer et al.

nicely review the impact of IFN-I induced BAFF and APRIL on B cell activation, class switching, and antibody production of plasma cells (Kiefer et al. 2012; Bencze, Fekete, and Pázmándi 2021).

In summary, the pleiotropic effect of IFN-I is a strength in antiviral immune response because it can activate numerous pathways to protect the human body against infection. Nevertheless, chronic secretion of IFN-I or too important dose of IFN-I can have severe negative impact on cells as mentioned below.



**Figure 10 – IFN-I effect on immune cells**

Innate sensor present in epithelial cells or innate immune cells will induce the production of IFN-I to have an autocrine or paracrine action. As IFN-I receptor (IFNAR) is ubiquitously expressed, IFN-I will impact every of the immune system. Here we represent the most described impact of IFN-I in the major immune cells of the innate and adaptive immune system.

## 17. IFN-I and its drawbacks

In mice, it has been shown in numerous studies that IFN-I control DCs turn over *in vivo*. IFN-I control the fine balance between pDCs apoptosis (Swiecki and Colonna 2015), or DCs apoptosis (Marraco et al. 2011), with hematopoietic stem cell (HSC) differentiation into DC (Essers et al. 2009). This turn over is important to replace exhausted DC present in the periphery during infections. Replacing DCs once they are mature and supposedly after they activate T cells, could prevent exacerbated immune response or immunopathology. However, when HSC are exposed for long period of time, IFN-I drastically decrease HSC survival *in vivo* (Essers et al. 2009), and severely impacts DC turn over.

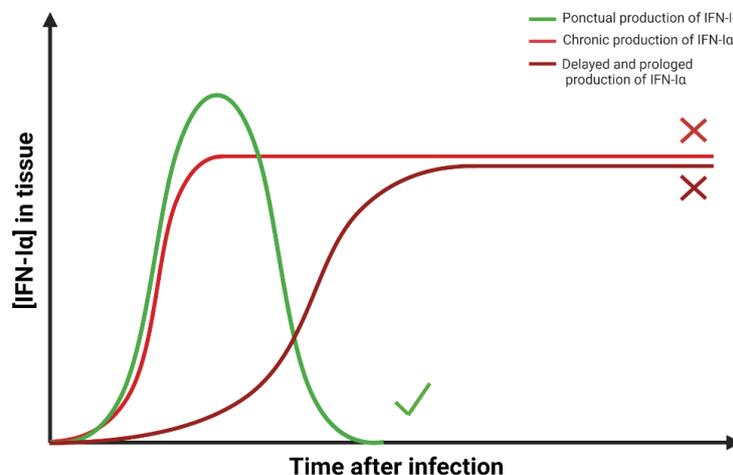
Human immunodeficiency virus 1 (HIV-1) can infect pDCs via their expression of CD4, CXCR4 and CCR5 (S. Patterson et al. 2001). Once infected, TLR7 present in the endosome of pDCs will be triggered by the presence of the virus (Beignon et al. 2005). Hence, during the acute phase of infection, pDCs will then start to produce high quantities of IFN- $\alpha$  and acquire APC properties. However, a chronic production of IFN- $\alpha$  can lead to immune dysfunction as it was observed that IFN- $\alpha$  expression was increased in secondary lymphoid organs (SLOs) of patients with progressive compared with non-progressive HIV-1 disease (Herbeuval et al. 2006). Another study demonstrated that upon HIV-1, pDCs were stuck in a partial state of activation where they only secrete IFN- $\alpha$ , and cannot continue their maturation to acquire APC properties (Beignon et al. 2005). These results highlight that pDCs could be the main sources of IFN- $\alpha$  production in HIV patient. The contradiction between the beneficial and deleterious effect of IFN- $\alpha$  in HIV-1 infection was nicely reviewed by *J.P Herbeuval et al.* and *M.L Gougeon et al.*. They discuss in these reviews the different mechanisms explaining IFN- $\alpha$  deleterious impact (Herbeuval and Shearer 2007; Gougeon and Herbeuval 2012; Tomasello et al. 2014). They demonstrated that IFN- $\alpha$  increases TRAIL expression on pDCs (Hardy et al. 2007a) and when interacting with CD4+ T cells induce T cell apoptosis. These reviews suggest that the chronic secretion of IFN- $\alpha$  may drive a chronic activation of immune cells leading to immune dysregulation and dysfunction.

With the recent coronavirus pandemic, many studies evaluated the importance of IFN-I and IFN-III in such infection. Mutation in IFN-I production and/or signaling pathways such as TLR3, IFNAR1, IFNAR2, IFNAR3, and IFNAR7, where described in patients with severe case of COVID-19 infection (Qian Zhang et al. 2020). These mutations abolished IFN-I production in patient's blood. The existence of neutralizing antibodies such as anti-IFN- $\alpha$  or anti-IFN- $\omega$ , was identified in 10% of patients with life-threatening cases of COVID-19, while only 0.33% of asymptomatic patients had these autoantibodies (Bastard et al. 2020). It is interesting to note that these patients who suffered of severe case of COVID-19 had complications with other infectious diseases. Their findings highlight the greater importance of IFN-I in SARS-Cov-2 infection compared to other infections. If IFN-I are needed at the early stage of infection, a prolonged exposure, in to strong quantities were reported in severe infections of SARS-Cov-2 (J. S. Lee and Shin 2020). Two studies shown that during infection, IFN- $\alpha$  in peripheral blood was maintained at high levels in patients with severe cases, whereas it decreases moderate symptoms patients (Lucas et al. 2020). An upregulation of ISG patient blood samples and in bronchoalveolar lavage fluid where described in severe COVID-19 (Zhu et al. 2020; Zhuo Zhou et al. 2020). This ISG upregulation was associated with an increase in proinflammatory genes. As SARS-CoV2 inhibit antiviral properties of IFN-I and IFN-III (Channappanavar and Perlman 2017), a dysregulated and delayed production of IFNs was observed, but TNF- $\alpha$ , IL-6 and IL-8, all proinflammatory cytokines, were produced before IFN-I and IFN-III and for a long period of time in severe cases of COVID-19 (Galani et al. 2021).

Kim et al. proposed a model explaining why IFN-I's role is contradictory in SARS-Cov-2 infection (Y.-M. Kim and Shin 2021). Upon respiratory epithelial cell infection SARS-Cov-2 will inhibit its recognition by innate sensors, block IFNs production and signaling so it can replicate freely in cell host. If epithelial cells cannot put in place an effective antiviral response innate immune cells, such as APC, will sense virus-infected cells and start to produce high level of IFN-I and/or IFN-III. IFNs will further activate surrounding immune cells and recruit other immune cells to the heavily infected tissue. This leads to a strong loop of amplification of IFNs

production that when associated with proinflammatory molecules can lead to strong side effects. Their hypothesis explains how IFNs delayed and exaggerated responses and its link with hyperinflammation contribute to the severe progression of COVID-19. Finally, while IFN-I enhance innate and adaptive immune defense against viruses, the time of exposure of IFN-I is crucial and needs to be tightly controlled [Figure 11].

In conclusion, IFN-I can induce beneficial and deleterious effect during infectious. It has been nicely reviewed by *E. Tomasello et al.* that several factors can modulate IFN-I response (Tomasello et al. 2014). Depending on IFN-I avidity for its receptor, the cell type stimulated by IFN-I modulate IFN-I response. The presence of other signal (TLR agonists or other cytokines) can also influence IFN-I response. But most importantly, the time of exposure, the duration of stimulation and the order of activation by another signal are crucial for IFN-I response. IFN-I response is complex and tightly regulated. To develop appropriate immunotherapies, all the previous parameters has to be taken into consideration for fear of inducing undesired adverse effects.



**Figure 11 – IFN- $\alpha$  appropriate immune response**

In green is represented the modelization of the appropriate IFN-I $\alpha$  secretion during the course of an infection. At the very early stage of infection, when viruses are recognized by PRR on epithelial cells or innate immune cells, IFN- $\alpha$  is heavily secreted by those same cells. However, there are inhibitory mechanisms, such as SOCS1, that will inhibit IFN- $\alpha$  signaling and production after a certain time. If IFN- $\alpha$  signaling and production are not stopped at the correct time deleterious effects can be observed on the immune response and favor virus replication. For instance, HIV block pDCs in their maturation at their IFN-I secretion stage, which participate to the maintaining of high level of IFN-I in tissues. This high level of IFN-I is correlated to disease progression. In SARS-Cov-2 infection, IFN- $\alpha$  is delayed and maintain for a prolonged time and is associated with severe case of COVID-19. In conclusion, the production of IFN-I have to be tightly regulated to induce a correct immune response.

## **B. IFN-III and antiviral responses**

### **18. IFN-III induce antiviral responses in epithelial cells**

As mentioned before, IFN-III induces almost similar pathways to IFN-I. It has been demonstrated that IFN-III stimulation induces PKR transcription even though this pathway is inhibited by orthopoxvirus (Bandi, Pagliaccetti, and Robek 2010). OAS and MxA are also activated by IFN-III stimulation, but at a lesser extent than IFN-I (Brand, Zitzmann, et al. 2005). The upregulation of these genes and proteins suggests that IFN-III can also inhibit viral protein synthesis, viral RNA degradation and can inhibit virions formation. The main difference between IFN-I and IFN-III will be the small range of epithelial cells and immune cells that respond to IFN-III in viral infections. Hence, studies focused on infections triggering hepatocellular cells, respiratory infections such as Influenza or SARS-Cov2, or gastrointestinal infections. By using HepG2 cell lines, a human liver cancer cell line, it was demonstrated that IFN-III induced the production of Core-binding factor subunit  $\beta$  (CBF $\beta$ ) protein (Xu et al. 2019). CBF $\beta$  protein is involved in the regulation of HIV replication (W. Zhang et al. 2012), but it also involved in the inhibition of HBV replication (Xu et al. 2019). Moreover, single nucleotide polymorphism (SNP) in *IFNL3* gene increases immune response to IFN- $\alpha$  and ribavirin treatment provided to HCV positive patients and help to eliminate HCV viruses (Ge et al. 2009; D. L. Thomas et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009). Concerning SARS-Cov-2 infection, It has been nicely demonstrated that IFN-L1 and IFN-L3 induce ISGs in the upper airways of infected patient (Sposito et al. 2021). This upregulation of ISGs was associated to protective antiviral response in moderate COVID-19 cases. They also demonstrate that respiratory epithelial cells were the main producer of IFN-L1, whereas DCs produced IFN-L2 and IFN-L3. These results support the protective role of IFN-III in SARS-Cov-2 infection, but are counterbalance with their observation that, IFN-L2 induces low ISGs and high p53 expression in severe COVID-19 cases.

## 19. IFN-III and their different impact on immune cells

Here will discuss the effect of IFN-III in different immune population and schematize it in **[Figure 12]**.

### **NK cells**

In mice, some evidence exists on the positive impact of IFN-III on NK cells after virus infection. IFN-III increases NK cell survival, proliferation and cytotoxic functions (Martinez, Huang, and Yang 2008; Paolini et al. 2015; Madera et al. 2016). Another study, demonstrated that murine NK cells express IFNLR1 gene, but at lesser extent than DCs (Fernando et al. 2015). No STAT1 phosphorylation was observed after IFN-III stimulation. Nevertheless, this same study showed that IFN-III enhanced the antimetastatic activity of wild type NK cells that was lost in IFNLR  $-/-$  NK cells. However, IL-28R $\alpha$  is not express by human NK cells, and IFN-III impact on human NK cells remains unclear (Krämer et al. 2011; Fernando, Young, and Smyth 2015). Hence, the observation in mice can be the results of cross-talk between DC and NK cells. Indeed, our lab demonstrate a cDC1/NK cells cross-talk in humans where NK cells are needed at early step of cDC1 antigen cross-presentation thanks to NK cell IFN- $\gamma$  and TNF- $\alpha$  production (Deauvieau et al. 2015). It could be suggested that IFN-III is involved in the cDC1/NK cell cross-talk in mice as well as in humans.

### **Macrophages**

A weak signal of IL-28R $\alpha$  is detected in human macrophages by flow cytometry (Yin et al. 2012) but no pSTAT1 was observed after IFN-III stimulation (Kelly et al. 2016). In humans, IFN-III can synergized with TLR8 pathways on monocyte-derived macrophages, to induce TNF- $\alpha$  and IL-12p40 (B.-S. Liu, Janssen, and Boonstra 2011). The same phenomenon is true with TLR4 that is increased by IFN-III stimulation, and enhance IL-12 production by macrophages (Groen et al. 2015). These results suggest an indirect effect of IFN-III on NK cells, as the superior secretion of IL-12 will act on NK cells and boost their own production of IFN- $\gamma$  (Groen et al. 2015). However this observation is in disagreement with the fact that when

PBMCs are culture with strong dose of IFN-III no expression of IFN- $\gamma$  is detected in NK cells (Krämer et al. 2011).

## **Neutrophils**

*IFN $\lambda$ R1* gene expression IL-28R $\alpha$  was described in human neutrophils, but only after TLR4 stimulation (Broggi et al. 2017). The authors described in mice that IFN-III induces ISGs expression but decrease their ROS production and degranulation capacity. The same observations remain to be elucidated in humans.

## **DC**

In mice, IFN-III stimulation enhance CCR7 expression at the surface of DC and enhance their migration to SLOs. However, in humans two papers highlight the induction of tolerogenic DC after IFN-III stimulation. In HCV infection IFN-III level in blood and liver are increased and leads to the upregulation of PD-L1 on cDCs (Dolganiuc et al. 2012) causing Treg expansion (Dolganiuc et al. 2012; Mennechet and Uzé 2006).

## **B cells**

A direct effect of IL-29 was observed on B cells with increase pSTAT1. Indeed, in the study of *M. Syedbasha et al.*, the authors demonstrated that IFN-III enhanced naive B cell differentiation into plasma cells via mTORC1 Pathway (Syedbasha et al. 2020). Interestingly, the same research team published previously that high IL-28B secretion negatively impact B cells proliferation and IgG production in favor to a Th1 response. The use of IL-28R $\alpha$  antagonist restored IgG secretion and induced a better greater influenza antibody after vaccination. This divergence can be explained by the use of IL-28B in their first paper (Egli et al. 2014), and then IL-29 in their second paper (Syedbasha et al. 2020). These divergences can also be caused by the presence of SNP is IL-28B that do impact the production of cytokines such as IL-4, IL-5 and IL-13 implicated in the Th1/Th2 balance. This IFN-III impact on the Th1/Th2 balance was also reported on several other publications (Dai et al. 2009; Jordan et al. 2007; Srinivas et al. 2008).

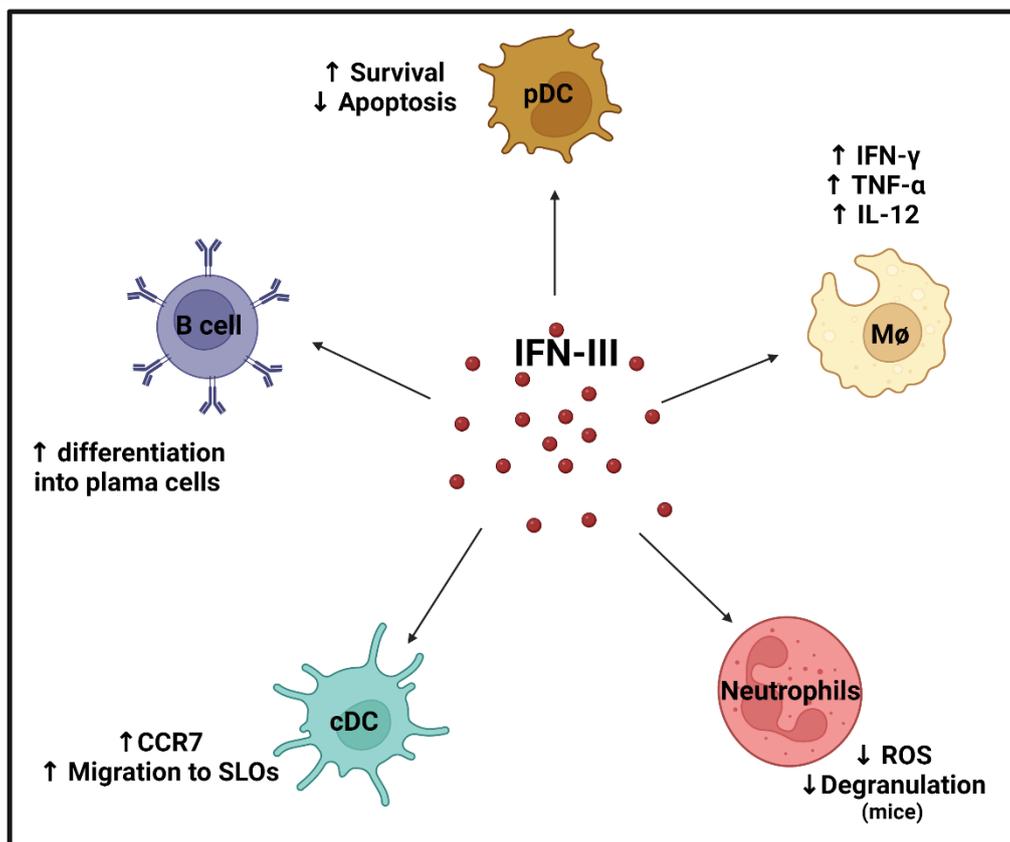
## VI. Focus on IFN-III stimulation on pDCs

One particular immune population that we did not yet talk about is pDCs. Several papers reported pDC responsiveness to IFN-III, and its impact on this populations. First, the IL-28R $\alpha$  expression by pDCs reported at steady state is weak but well present at their surface (Kelly et al. 2016). IL-28R $\alpha$  expression at steady state on pDCs was also reported by *N.Megjugorac et al.* but they show that IL-28R $\alpha$  is express at lower level on pDCs compared with total PBMCs, and with the whole population of DCs (Megjugorac, Gallagher, and Gallagher 2009). The expression of IL-28R $\alpha$  receptor in cells can be discuss as the provided tools for flow cytometry do not allowed an optimal analysis. By flow cytometry the MFI increase is too weak to be correctly interpreted. The same is true for the analysis of IL-28R $\alpha$  expression by RT-PCR. However, when cells are activated with viruses, IL-28R $\alpha$  level increase sufficiently on pDCs which confirmed that pDCs upregulate IL-28R $\alpha$  expression after viral activation (Yin et al. 2012). Phosphorylation of pSTAT1 is often used as a surrogate marker of IFN-III response. Upon IFN-III stimulation a significant increase of pSTAT1 is observed in pDCs along with pSTAT3 and pSTAT5 (Kelly et al. 2016). IFN-III also induce ISGs transcription, as they increase PKR and CXCL10 expression (Kelly et al. 2016). Indeed, in response to IFN-III pDCs increase their production and secretion of IFN-I $\alpha$ , TNF- $\alpha$ , and CXCL10 (Finotti et al. 2016). Moreover, the upregulation of activation markers such as CD80, CD86, CD83, CD123, HLA-DR are observed on pDCs following IFN-III stimulation [Table 5] (Megjugorac, Gallagher, and Gallagher 2009; Finotti et al. 2016) .

Table 5: Regulation of marker by IFN-III on pDCs		
Upregulation or Downregulation	Activation Marker	References
↑	CD80	(Megjugorac, Gallagher, and Gallagher 2009)
↑	CD86	(Megjugorac, Gallagher, and Gallagher 2009) (Finotti et al. 2016)
↑	CD83	(Megjugorac, Gallagher, and Gallagher 2009) (Finotti et al. 2016)
↑	ICOS-L	(Megjugorac, Gallagher, and Gallagher 2009)
↑	CCR7	(Megjugorac, Gallagher, and Gallagher 2009)
↑	CD62L	(Megjugorac, Gallagher, and Gallagher 2009) (Finotti et al. 2016)
↑	CD123	(Finotti et al. 2016)
↓	CD303	(Finotti et al. 2016)
↑	HLA-DR	(Finotti et al. 2016)

In terms of functionality, IFN-III increases pDCs survival after 18h and 42h of culture without IL-3 (Finotti et al. 2016). For *in vitro* culture of human pDCs, IL-3 is generally added to prevent pDCs from dying. Finally, as for IFN-I, IFN-III protects pDCs from Dexamethasone-induced apoptosis (Finotti et al. 2016).

Even though these studies provided meaningful information on pDCs response to IFN-III and its functionality, there is not enough knowledge considering that pDCs are APCs, and the main producers of IFN-I, a crucial cytokine in antiviral and antitumor responses. IFN-III impact was strongly studied on epithelial cells, but further studies shall be performed on its impact on pDCs.



**Figure 12 – Specific Immune response induced by IFN-III**

Unlike IFN-I that act on every cell, IFN-III has a smaller range of action on immune cells. IFN-III increase pDCs survival and protects them against apoptosis. IFN-III induce B cell differentiation into plasma cells. It upregulates CCR7 expression on cDCs enhancing their capacity to migrate to SLOs. On MØ IFN-III will upregulate their production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-12. Finally, it was described in mice, that IFN-III inhibit neutrophils functions by decreasing their ROS production, and degranulation capacities.

## VII. Antitumor Effects of IFNs

### A. IFN-I in antitumor immunity

Nowadays, the immunostimulatory role of IFN-I in antitumor immunity have been well documented.

First, IFN-I have a direct effect on cancer cells by inducing apoptosis and by blocking their cell cycle. Indeed, it has been demonstrated that in human colorectal cell line (KM12L4), IFN-I induce apoptosis through the activation of procaspase 3, 8 and 9 (Choi et al. 2003; Thyrell et al. 2002). IFN-I could also allow the release of cytochrome C by mitochondria leading to cell apoptosis (Choi et al. 2003). By using small interfering RNA (siRNA), cFLIP, caspase-8 and DR5 were identified as major players in the apoptosis pathways activated by IFN-I in WISH cancer cell line. As in antiviral response, IFN-I will induce ISG, as PKR and OAS, to inhibit the cellular machinery of cancer cells leading to cell death. Actually, the role of PKR in cancer development is controversial because cancer cells can hijack PKR function in favor of tumor progression (Pataer et al. 2009). IFN-I will also slow the cell cycle of cancer cells as it was reported in breast (Balkwill, Watling, and Taylor-Papadimitriou 1978) and prostate (Hobeika, Subramaniam, and Johnson 1997) cancer cell lines.

Second, IFN-I will increase tumor recognition by the immune system. In human breast and ovarian cancer, IFN-I will increase MHC-I expression of cancer cells (Boyer et al. 1989). This could lead to an increase interaction with tumor-specific CD8+ T cells and permit cancer cells elimination. Unfortunately, cancer cells developed mechanisms to block their expression of MHC-I, by dysregulating NF-K $\beta$  pathways, blocking ISG transcription, or antigen processing, in order to avoid CD8+ T cells detection (Cornel, Mimpen, and Nierkens 2020).

## **B. IFN-I as immunotherapy**

Due to its pro-apoptotic and anti-proliferative properties on cancer cell, IFN-I were approved by the FDA to treat carcinoma, melanoma, leukemia, and sarcoma (Pasquali and Mocellin 2010). However, as IFNAR is ubiquitously expressed across the human body, high toxicity was associated with cancer treatment with IFN- $\alpha$ 2b (Kirkwood et al. 2002). Patients treated with IFN- $\alpha$ 2b developed flu-like symptoms, articular pain, psychic disorders, and neurotoxicity. All these severe side effects forced patient to stop IFN- $\alpha$ 2b treatment beforehand. Therefore, it is important to find new immunotherapies inducing a similar immune response with less toxicity. This is where IFN-III could become an interesting target as its range of responding cells is restricted.

## **C. IFN-III in antitumor immunity**

IFN-III being the most recently discovered cytokine family, its effects in antitumor immunity are not yet fully understood. Important characterization of IFN-III's role in the TME has been done in mice, but the same is not true in humans. In humans, some direct IFN-III activity over cancer cell line has been demonstrated but no studies investigated the role of IFN-III on tumor-associated immune cells.

### **20. In mice**

In mice several studies focus on IFN-III direct effect and demonstrated anti-angiogenic, anti-proliferative, anti-apoptotic properties, along with cell cycle inhibition, and an increase of MHC-I by cancer cells. First, BW5147 lymphoma cells were transfected with the IL-28R $\alpha$  chains and stimulated with different doses of IFN- $\lambda$ 1. While the proliferation of non-transfected cell was not affected, BW5147 cells expressing IL-28R $\alpha$  had a dose-dependent inhibition of proliferation (Dumoutier et al. 2004). In 2006, IFN-III inhibition of cancer proliferation was also demonstrated in B16 melanoma cells (Lasfar et al. 2006). In this study, the authors developed B16 cells constitutively expressing murine IFN- $\lambda$ 2 and injected them *in vivo*. Tumor development was

heavily retarded or completely abrogated when mice received B16-IFN- $\lambda$ 2 cells. In addition of inhibited proliferation, angiogenesis was also decreased in tumor that still developed (Lasfar et al. 2006). *In vitro*, B16-IFN- $\lambda$ 2 cells had a significant upregulation of MHC-I after 3 days of cultures. These results were supported by another study showing that B16 cell transduced with IFN- $\lambda$  have an increased expression of MHC-I, but also in caspase 3/7 (Sato et al. 2006). *Sato et al.* also shown that p21 increases in B16 expressing IFN- $\lambda$ 2, resulting in cell cycle arrest. Finally, LA795 lung adenocarcinoma cells transfected with IFN- $\lambda$ 2 shown a decreased tumor growth through inducing cancer cell apoptosis (Yulan Yan et al. 2013).

If IFN-III has a direct impact on tumor growth, it was also reported that IFN-III could activate NK cells, T cells and neutrophils to eradicate cancer cells. In mice injected with luc-colon26 cells in the liver, an increase of NK and NKT cells were observed among hepatic lymphocytes. In untreated mice, NK and NKT cells represented only 1% compared with 10% of infiltration in mice treated with IFN- $\lambda$ 2 (Sato et al. 2006). When exposed to IFN- $\lambda$ 2 NK and NKT cells had a significant increase of their killing activity against luc-Colon26 cells. Increased infiltration of NK cells within tumors in presence of IFN- $\lambda$ 2 transfected cancer cell or after IFN- $\lambda$  injection was also reported in other papers (Numasaki et al. 2007; Abushahba et al. 2010; Yulan Yan et al. 2013; Fernando et al. 2015). *Fernando et al.* demonstrated that IFN- $\lambda$  injection in mice with B16 tumors promoted antimetastatic activity of WT NK cells and suggest that IFN- $\lambda$  can directly regulate NK cell effector functions.

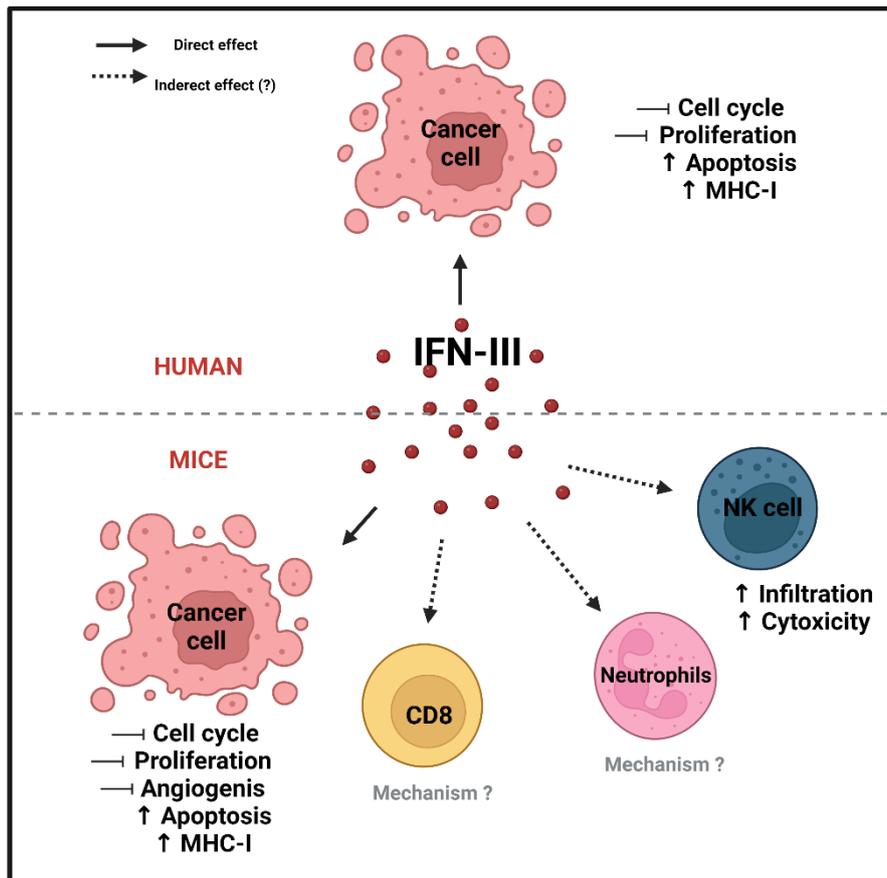
To determine which immune cells respond to IFN-III in the TME, *Numasaki et al.* depleted CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells and neutrophils in mice carrying fibrosarcoma cells expressing IL-28B. Depletion of CD8<sup>+</sup> T cells and NK cells inhibit the tumor antiproliferation role of IL-28B (Numasaki et al. 2007). CD4<sup>+</sup> T cell depletion did not counteract antitumor action of IL-28. It is important to notice that depletion of neutrophils partially abrogated IL-28B antitumor activity. These results highlight the impact, rather direct or indirect, of IFN-III on CD8<sup>+</sup> T cells, NK cells and neutrophils in mice. The impact of IFN-III on T cell antitumor function was also demonstrated in other publications (Yulan Yan et al. 2013; Burkart et al. 2013).

## 21. In humans

Even if numerous studies demonstrated the role of IFN-III in antitumor immune response in mice, only few studies focus on IFN-III antitumor function in humans.

HT29, a colorectal cancer line responding to IFN-III was intensively used to assess IFN-III antitumor functions. As in mice, an increase of MHC-I was observed in HT29 cell line when exposed to IFN-III (Lasfar et al. 2006; Li et al. 2008). An induction of apoptosis was also observed in IFN-III stimulated HT29 cells (Li et al. 2008; Hui et al. 2011). IFN-III induced-apoptosis was also observed in lung adenocarcinoma cell lines (Tezuka et al. 2012). Moreover, HT29 cells incubated with IFN-III showed G1/G0 phase cell cycle arrest (Li et al. 2008). Anti-proliferation properties of IFN-III were also validated in numerous human cancer cell line (Zitzmann et al. 2006; Hui et al. 2011; Tezuka et al. 2012). Finally, the only evidence of IFN-III impact on tumor associated immune cells was provided by our team. We showed that IFN-III induced a Th1 polarization when added to culture of tumor associated immune cell suspension (Hubert et al. 2020).

Altogether, these results demonstrate that, as IFN-I, IFN-III can induce apoptosis of cancer cells, stop their proliferation, arrest their cell cycle and increase their expression of MHC-I for a better immune response. Unfortunately, to this day, no further studies focus on tumor associated immune cell in humans and their response to IFN-III. More than just assessing which immune cells respond to IFN-III, further studies need to be performed to understand IFN-III's role in human antitumor functions.



**Figure 12 – Antitumor function of IFN-III in mice and humans**

In mice to role of IFN-III in antitumor function has been quite investigated. IFN-III has direct effect on murine cancer cells. IFN-III inhibits the cell cycle, the proliferation, and angiogenesis of mice cancer cells. IFN-III also upregulates MHC-I on mice cancer cells and increase their apoptosis. IFN-III antitumor activity is dependent of the presence of CD8+T cells, neutrophils and NK cells. However, it is not yet determined whether IFN-III act on directly on these cells or if it is an indirect effect.

## VIII. Differences between IFN-I and IFN-III

We presented the role of IFN-I and IFN-III in antiviral and antitumor immune response and how they engage similar signaling pathways in similar cells. These observations raised the question of what would be the interest of these strong redundancies between IFN-I and IFN-III? In other terms, is there any differences between IFN-I and IFN-III? Different studies tackled this question through different approaches.

The first main difference was the level of pSTAT induced by IFN-I and IFN-III. Where IFN-I induced a strong level of pSTAT, IFN-III induced a weaker signal in epithelial and immune cells [Table 6] (Sheppard et al. 2002; Kotenko et al. 2002; Yin et al. 2012; Kelly et al. 2016).

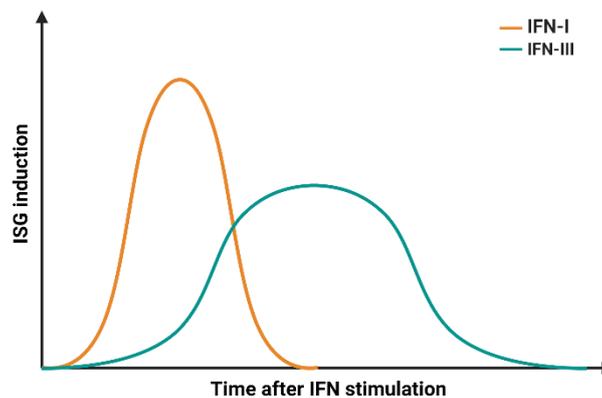
	IFN-I	IFN-III
pSTAT1	++	+
pSTAT2	++	+
pSTAT3	++	+
pSTAT4	∅	∅
pSTAT5	++	+
pSTAT6	++	∅

**Table 6 : Differential phosphorylation of STAT between IFN-I and IFN-III**

The canonical JAK/STAT pathways of IFN activation is shared between IFN-I and IFN-III, but studies demonstrated IFN-III antiviral properties are dependent of MAPKs pathways. Human colon carcinoma-derived cell line T84 were infected with vesicular stomatitis virus (VSV) and treated with IFN-I or IFN-III in presence of ERK inhibitor (Pervolaraki et al. 2017). While IFN-I control VSV infection in T84, IFN-III did not when ERK was inhibited. When Raji cells were stimulated with IFN-III, the expression of ISGs were completely lost in presence of JNK and p38 inhibitors, two protein involved in MAPK pathways (Zhangle Zhou et al. 2007). ISGs expression were not lost when Raji cells were treated with IFN-I. These results demonstrate that MAPK pathways is essential for a correct induction of ISGs by IFN-III and a proper control of viral load. This statement is not true for IFN-I.

A lower dose of IFN-I will induce a more potent ISG induction compared to IFN-III. In Raji cells, 1 µg/mL of IFN-α will induce a 150 fold increase of ISG56, where 1 µg/mL of IFN-λ will only induce a 5 fold increase (Zhangle Zhou et al. 2007). Even at the excessive dose of 1 mg/mL of IFN- λ, ISG56 fold increase will only reach 25. In B cells, 100 ng/mL of IFN-α induce ~70% of MxA+ B cells, where 100 ng/mL of IFN-λ induces only 35% MxA+ B cells.

IFN-I response is then more potent, especially at lower doses and induce ISGs at an earlier time compared with IFN-III [Figure 14]. The maximum transcription of ISGs when Raji cells are stimulated with IFN-α is reached at 8h post-treatment, against 16h for IFN-λ (Zhangle Zhou et al. 2007).



**Figure 14 – Differential ISG induction by IFN-I and IFN-III**

The orange curve represents the schematize induction of ISGs after IFN-I stimulation and the cyan curve represents the schematize induction of ISGs after IFN-III. Both in epithelial cells. IFN-I induced ISG expression is stronger and happens earlier than IFN-III induced ISG expression.

Finally, a very nice paper compared the transcription program of IFN-I or IFN-III stimulated human colon organoids (Pervolaraki et al. 2017). Even though the same ISG are activated between IFN-I and IFN-III, the kinetics of their activation is very different. IFN-β appears to activate 4 different waves of ISGs at 3h, 6h, 12h, and 24h post-treatment, when IFN-λ seem to induce the majority of its ISG transcription at 24h post treatment.

In summary, the main differences between IFN-I and IFN-III do not rely on the induction of differential ISGs. Their differences rely on 1) the range of cells they can respectively activate, 2) the intensity of activation signal, and 3) their kinetics of activation. IFN-III act on a few cells, and induced a weaker activation signal that is delayed compared to IFN-I. Nevertheless, these characteristics are key in the development of new immunotherapies. A small range of action goes with less side effects and a decreased toxicity, and a weaker activation signal underlines a less aggressive treatment. Therefore, IFN-III is a great target for the development of new immunotherapies, and investigation shall continue to better characterizes its function on epithelial and immune cells.

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## **RATIONALE AND HYPOTHESES DRIVING PHD STUDIES**

### *(OBJECTIF DE LA THESE)*

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Our team previously demonstrated that cDC1 were the only immune cells secreting IFN-III in the TME. This IFN-III secretion was corelated with a good clinical outcome (Hubert et al. 2020). In the light of all the information gathered in the introduction, the role of IFN-III has been well investigated in the context of viral infection, and on human cancer cell line. Nevertheless, no studies focus on the role of IFN-III antitumor immune response in human. Our research was also motivated by the need to develop new immunotherapy strategies as past and current ones have a low response rate (i.e. ICI) or are associated with too severe side effect to be accepted as routine treatment (i.e. IFN- $\alpha$ 2, TLR agonists and DC based vaccines). As IFN-III shares similar function with IFN-I but with a limited ranged of action on immune and epithelial cells, less toxicity would be caused by IFN-III treatment of cancer patient. Thus, IFN-III represent an interesting target for the development of new immunotherapies.

My PhD project is therefore focused on **the study of the role of IFN-III in antitumor immunity** to answer several questions that have remained unanswered:

- Which immune cell do respond to IFN-III in blood and in tumors?
- What is the effect of IFN-III on those cells?
- What would be the beneficial impact of IFN-III treatment over tumor-associated immune cells?

To answer these questions, we study immune cells from blood and tumor samples using flow cytometry technics, mIF, multiplex assay and bioinformatic tools. Our research results are explained in the following part and in a paper entitled "IFN-III prime pDCs for TLR7 activation and unleash pDCs function in tumors.". They provide crucial response elements for the understanding of mechanisms activated by IFN-III in tumor micro-environment.



# RESULTS



## **IFN-III prime pDCs for TLR7 activation and unleash pDCs function in tumors.**

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### **Abstract**

Interferons are central players in antiviral and antitumor immune responses through their direct effects on infected or tumor cells, but also on immune cells. While the positive impact of Type I Interferons (IFN-I) on cancer development is well understood, the role of Type III interferons (IFN-III) in the tumor microenvironment (TME) remains unclear. Using flow cytometry and RNA sequencing analysis, we demonstrate that plasmacytoid dendritic cells (pDCs) are the immune cells that respond best to IFN-III in blood and tumors. We observed in blood, that IFN-III increase pDCs activation molecules such as CD80, CD86, and HLA-DR, as well as PD-L1 and ICOS-L. IFN-III also increases TLR7 expression on pDCs, enhancing their capacity to respond to Imiquimod (IMQ). Indeed, pDCs pre-treated with IFN-III produce more IFN- $\alpha$  upon TLR7 activation compared to non-pre-treated pDCs. Finally, TGF- $\beta$  is involved in the inhibition of IFN- $\alpha$  by pDCs in the TME, we finally demonstrated that IFN-III can overcome TGF- $\beta$  inhibition on pDCs by restoring their production of IFN- $\alpha$ . Our findings indicate that targeting tumor-associated pDC with a combination of IFN-III and TLR7-L to restore their IFN- $\alpha$  production might be a strategy to induce antitumor immunity.

### **Key words**

Plasmacytoid Dendritic cells (pDCs), Type III Interferons (IFN-III), Toll-Like Receptor 7 (TLR7), Transforming Growth Factor Beta (TGF- $\beta$ ), Type I interferons (INF-I)

## **Abbreviations**

cDC: classical DC ;  
DC: dendritic cells ;  
DEG : Differentially Expressed Gene ;  
GSEA: gene set enrichment analysis ;  
IFN-I: type I interferons ;  
IFN-III: type III interferons ;  
IFNAR: IFN-alpha receptor ;  
IFNLR: IFN-lambda Receptor  
IL: interleukine ;  
IMQ : Imiquimod ;  
ISRE: interferon-stimulated response elements  
ISG: IFN-stimulated gene ;  
LC: Langerhans cells,  
mIF: multiplex-Immunofluorescence  
PBMC: peripheral blood mononuclear cells ;  
PCA : Principal Component Analysis ;  
pDCs: plasmacytoid DC ;  
TA-DC: tumor-associated DC ;  
TA-pDCs: Tumor-Associated pDCs  
TGF- $\beta$ : Transforming Growth Factor Beta ;  
TLR: toll-like receptor ;  
TME: tumor microenvironment ;  
TNF- $\alpha$  : Tumor necrosis factor alpha (TNF- $\alpha$ )  
SN-Dil : supernatant from primary dilacerated tumors

## Introduction

pDCs can be separated from conventional dendritic cells (cDC) thanks to their lack of CD11c and the expression of BDCA2/CD303 and IL-3 Receptor (CD123) at their surface. Their main function is to produce high amount of IFN-I after TLR7 and TLR9 activation by viral ssRNA and ODN CpG respectively (Cella et al. 1999; Siegal et al. 1999). IFN-I produced by pDCs will induce an antiviral and antitumor responses in epithelial cells and immune cells (Gresser and Belardelli 2002; Zitvogel et al. 2015). These observations led to the approval of IFN-I, more precisely IFN- $\alpha$ 2A and IFN- $\alpha$ 2B, as a treatment of several malignancies (Asmana Ningrum 2014). An IFN-I signature in the TME correlates with “hot tumors” phenotype that is characterized by a strong immune infiltrate and a better responsiveness to immunotherapies. Conversely, the downregulation of IFN-I receptor (IFNAR1) is associated with a poor clinical outcome and tumor progression (Katlinski et al. 2017; Castiello et al. 2018) in colorectal carcinoma (Katlinski et al. 2017) and melanoma (Sprooten, Agostinis, and Garg 2019).

The precise role of pDCs within the tumor microenvironment (TME) remains unclear. pDCs infiltration within tumor has been correlated with positive prognosis in colorectal (Kießler et al. 2021), breast (Michea et al. 2018; Oshi et al. 2020) and pancreas cancers (Plesca et al. 2022). However, pDCs were associated with a negative prognosis in ovarian (Labidi-Galy et al. 2011; 2012) and breast cancers (Treilleux et al. 2004; Sisirak et al. 2012). This discrepancy may result from their dysregulation in the TME, in particular their decreased capacity to secrete IFN- $\alpha$  (Hartmann et al. 2003). After TGF- $\beta$  exposure a lower TLR9 expression has been noticed (Bekeredjian-Ding et al. 2009), IRF7 expression is reduced and its translocation to the nucleus is inhibited (Sisirak et al. 2013). All three elements lead to the inhibition of IFN- $\alpha$  production.

In parallel, our team has recently shown that the cDC1 population contributes to antitumor responses through production of Type III interferon (IFN-III) (also known as IFN- $\lambda$ ). Primary breast tumors and publicly available transcriptomic data associated cDC1 production of IFN-III with favorable patient outcomes. IFN-III family is composed of IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3, also respectively named IL-29, IL-28A and IL-28B. IFN-III first interacts with the IFN $\lambda$ R1 chains (Sheppard et al. 2002), that recruit the IL-10R $\beta$  chain (Yoon et al. 2010) to form the heterodimer IFN-Lambda Receptor (IFNLR). Once IFN-III interacts with IFNLR, the intracellular signaling pathway is similar to the IFN-I pathway. JAK1 and TYK2 tyrosine kinases induce STAT1 and STAT2 phosphorylation which will both bind to IRF9 and create the ISGF3 complex (Au-Yeung, Mandhana, and Horvath 2013). ISGF3 translocate to the nucleus where it binds interferon-stimulated response elements (IRSE) to activate the transcription of several interferon-stimulated genes (ISGs).

The Type I IFN (IFN-I) receptor (composed of IFNAR1/2 subunits) is ubiquitously expressed in human whereas the expression of IFN $\lambda$ R1 is not well documented in immune cells from the TME. To better understand the respective role of cDC1 and pDC in the TME, we investigated the impact of IFN- $\lambda$ 1 on tumor-associated (TA) immune cells. We first observed a high expression of IFN $\lambda$ R1 on pDC. IFN-III strongly upregulates TLR7 on pDCs and enhance pathways involved in IFN-I production. More importantly, we observed a high capacity of IFN-III to prevent the inhibitory effect of TGF- $\beta$ . Those results will allow us to propose new immunotherapies enhancing pDCs immunostimulatory.

## Results

### pDCs strongly respond to IFN-III.

To evaluate pDCs response to IFN-III, we first investigated the expression of IFN $\lambda$ R1 and IL-10R $\beta$ , the two chains of IFNLR, by looking at public RNA sequencing data from the Human Cell Atlas. pDCs are the immune cells with the highest RNA level of IFN $\lambda$ R1 (Fig. 1A), and the third immune cell population with the highest mRNA level of IL-10R $\beta$  (Fig. 1B). B cells and cDC1 also express a small amount of IFN $\lambda$ R1 mRNA. Then, we evaluated STAT1 phosphorylation (pSTAT1) in response to IFN-I or IFN-III stimulation on several immune cells from Peripheral Blood Mononuclear Cells (PBMCs) (Fig. 1C-D). pDCs were the only cells with a significant pSTAT1 increases when stimulated with IFN-III. Moreover, pSTAT1 in pDCs after IFN-III stimulation was as strong as the one induced with IFN-I stimulation. Of note, a slight increase in pSTAT1 MFI on B cells was observed. This data recalls the work of *Syedbasha et al.* where an increase in pSTAT1 can be observed in IFN-III stimulated B cells. In their study they demonstrated that IFN-III enhanced naive B cell differentiation into plasma cells via mTORC1 Pathway (Syedbasha et al. 2020). Except for pDCs and B cells, neither cDCs (Fig. 1C-D), T cells nor NK cells responded to IFN-III (Supplementary Fig. 1A-C). Thus, pDCs from PBMCs are the immune cells with the strongest respond to IFN-III.

### IFN-III increase activation marker expression on pDCs, along with PD-L1 and ICOS-L expression

As pDCs strongly respond to IFN-III stimulation, we evaluated IFN-III ability to induce phenotypic modifications on pDCs. Thus, we purified pDCs from PBMCs and incubated them 24h with IFN-III or IL3 which is a classically used survival cytokine for human pDC. We observed that pDCs cultivated with IFN-III had a significant viability increase after 24h of culture, reaching 75% against 40% in the control condition (Fig. 2A). Interestingly, IFN-III can

induce a survival as important as IL-3. In addition, IFN-III increases the expression of activation markers such as CD80, CD86, and HLA-DR (Fig. 2B). However, pDCs are known to have a dual role in immune responses as they secrete high amount of IFN- $\alpha$  to activate immune cells. Thus, we assessed their expression of immunoregulatory molecules PD-L1 and ICOS-L. Both proteins were upregulated after IFN-III stimulation. Around 80% and 42,5% of pDCs expressed ICOS-L or PD-L1 respectively after IFN-III stimulation (Fig. 2C-F). Interestingly, it seems that two groups emerged through PD-L1 expression upon IFN-III stimulation. We can observe a group of healthy donors with a high expression of PD-L1 (reaching 80% of pDC) and a group with a low expression of PD-L1 (around 20-30%). As PD-L1 is strongly increased after IFN-III stimulation, we will use PD-L1 as a surrogate marker to evaluate pDC responsiveness to IFN-III in the following experiments. These results highlight the potential immunostimulatory role of pDCs once stimulated with IFN-III, as well as their possible immunomodulatory role in immune responses.

### **IFN-III induces TLR7 and pathways involved in IFN-I production**

To go further on the characterization of IFN-III effect on pDCs, we performed bulk RNA-sequencing on pDCs from PBMCs stimulated with or without IFN-III. We first determined the best timepoint of analysis corresponded to 12h post-treatment (Supplementary Fig. 2A). We observed by RT-qPCR that pDCs had the higher expression of OAS2 mRNA, a known ISG, after 12h of culture with IFN-III compared to 6h or 18h of stimulation. Principal component analysis (PCA) on our dataset revealed that IFN-III stimulation explained 45% of the variability between our samples (Fig. 3A). A gene expression analysis showed that IFN-III had an important effect on pDCs as 1489 differentially expressed genes (DEGs) were upregulated, and 888 were down-regulated when comparing IFN-III-stimulated pDCs with non-stimulated pDCs (Fig. 3B). The upregulation of *CD274* gene was observed among DEGs, supporting our previous observation of an upregulation of PD-L1 with IFN-III at the protein level. Other genes such as *APOL6*, *IFIT2*, *SOCS1* involved in the STAT pathways were highlighted in this graph, but also *TTC38* and *MAK* involved in proliferation and mobilization respectively. *NLCR5* is the gene with the strongest adjusted p-value, and is involved in the transcriptional regulation of MHC-I. However, in order to investigate which pathways were enriched in IFN-III stimulated-pDCs compared to non-treated pDCs, we performed a Gene Set Enrichment Analysis (GSEA) (Fig. 3C). The hallmark gene set with the most important normalized enrichment score (NES) is the interferon gamma response. In addition, interferon alpha response was the hallmark with the most elevated percentage of enriched genes. These two hallmarks appear together in this GSEA because they have numerous genes in common. A Venn diagram demonstrated that

almost half of the enriched genes of those 2 hallmarks were common ones ([Supplementary Fig. 2B](#)).

PI3K-AKT-mTOR signaling and mTORC1 signaling are two hallmarks enriched in IFN-III stimulated-pDCs ([Fig. 3D](#)) and are involved in the production of IFN-I. Indeed, it has been well demonstrated that upon TLR7 activation, mTORC1 interacts with TRAF3 and TRAF6 resulting in the secretion of IFN- $\alpha$  (Saitoh et al. 2017). The complex formed with TRAF3 and TRAF6 activates IRF7 which with the help of PI3K pathways will translocate to the nucleus to induce IFN-I $\alpha$  transcription (Bao and Liu 2013; Cao et al. 2008). When we studied the mRNA expression of *TLR7* and *IRF7* genes, both belonged to the top 50 DEGs between IFN-III-stimulated versus non-stimulated pDCs ([Fig. 3D](#)).

Altogether these results suggest that IFN-III stimulation will prime pDCs to better respond to TLR7 ligands and to produce a stronger IFN-I response.

### **IFN-III pre-treatment sensitizes pDCs to low dose of a TLR7 agonist**

First, we observed that TLR7 mRNA increased expression ([Fig. 4A](#)) was also linked to an increase expression of TLR7 at the level. TLR7 protein expression on pDCs was strongly increased after IFN-III treatment compared to non-treated pDCs (p-value = 0.008) ([Fig. 4B-C](#)). As TLR7 expression was increased, we wondered if other TLRs would also be increased after IFN-III treatment ([Supplementary Fig. 3A](#)). Interestingly TLR9 was not affected by IFN-III treatment, (RNA and protein level) ([Supplementary Fig. 3B-C](#)). We also observe a significant increase of *RIG-I* gene expression, a cytosolic pattern recognition receptor (PRR) responsible for IFN-I response, and *MDA5* gene, a PRR known to induce antiviral responses ([Supplementary Fig. 3A](#)).

We then investigated if IFN-III pre-treatment on pDCs would enhance their responsiveness to a TLR7-ligand such as Imiquimod (IMQ). By observing PD-L1 and ICOS-L expression on pDCs treated with different doses of IMQ, we determined a suboptimal dose of IMQ, which corresponds to 50 ng/mL ([Supplementary Fig. 4A-B](#)). When using a strong concentration of IMQ (250 ng/mL), no difference over PD-L1 expression was observed when comparing IFN-III-stimulated pDCs to non-stimulated pDCs ([Fig. 4D-E](#)). However, when using the suboptimal concentration of IMQ, PD-L1+ and ICOS-L+ pDCs were strongly upregulated with IFN-III pre-treatment. Only 5% and 10% of pDCs in the control expressed PD-L1 or ICOS-L, against 60% and 80% respectively in IFN-III pre-treated pDCs ([Fig. 4D-E](#)). Besides, we observed an HLA-DR MFI higher in IFN-III pretreated pDCs (~40 000 units) compared to non-treated pDCs in suboptimal dose of IMQ (~15 000 units) ([Supplementary Fig. 4C](#)). Finally, with the strong dose

of IMQ we observed that IFN- $\alpha$  secretion has been multiplied by 25 when pDCs were pre-treated with IFN-III compared to no pre-treatment (Fig. 4F).

Altogether those results demonstrate that IFN-III prime pDCs for a better response to IMQ.

### **IFN-III stimulated pDCs acquire an IFN-I secretion phenotype upon TLR-7 stimulation**

It has been demonstrated that following pDC activation, the presence of different pDC subpopulations emerge. For example, three subsets have been characterized by their expression of PD-L1 and CD80 (Alculumbre et al. 2018). A PD-L1+ CD80- pDCs, named P1 was associated with the high secretion of a wide range of cytokines. The P3 subpopulation characterized as PD-L1- CD80+, was associated with adaptive function as they can activate T cells. The last subpopulation P2 which is PD-L1+ CD80+, appears to be an intermediate group able to secrete IFN-I and to activate CD4+ T cells. Thus, we pre-treated them with IFN-III or IL-3 before stimulating them with IMQ (250 ng/mL) (Fig. 5A). 65% of IFN-III pre-treated pDCs were PD-L1+ CD80- (P1 subpopulation) after IMQ stimulation, whereas IL-3 pre-treated pDCs had only 10% of cells belonging to P1 (Fig. 5B-C). When pDCs were pre-treated with both IFN-III and IL-3, P1 subpopulation reach 30%. These experiments show that cultivating pDCs with IFN-III will polarize pDCs towards a P1 subpopulation specialized in secretion of cytokines secretion. This was confirmed by high amount of IFN- $\alpha$  detected in IFN-III pre-treated pDC supernatants (25 ng/mL against 8ng/mL with IL3 pretreatment in response to IMQ) (Fig. 5D).

PD-L1+ CD80- pDCs generated by IFN-III pre-treatment also secrete high dose of IFN- $\beta$ , CX3CL1, IL-6, and TFN- $\alpha$  compared with IL-3 in response to IMQ (Fig. 6A-E). On the opposite, CCL19 and CXCL10 secretion, two cytokines implicated in T cells recruitment, were increase when pDCs were pre-treated with IL-3 which is consistent with a specialization towards a P3 subpopulation and T cell activation (Fig. 6F-G).

In conclusion, IFN-III will favor pDC secretion of cytokines and chemokines triggering innate immune cells, whereas IL-3 induces the secretion of cytokines and chemokines directed against T cells.

### **Tumor-associated pDC strongly respond to IFN-III**

pDCs are the main producer of IFN-I, a key cytokine in antitumor immune response, but within the TME pDCs lose this capacity. Accordingly, to our results from blood pDCs, we made the

hypothesis Tumor-associated pDCs (TA-pDCs) may also respond to IFN-III. To validate this hypothesis, we first screened immune cell of the TME for their expression of IFN $\lambda$ R1 and IL-10R $\beta$  chains. We used public transcriptomic data available on Tumor Immune Single-cell Hub (TISCH) to study IFN $\lambda$ R1 and IL-10R $\beta$  mRNA level (Fig. 7A-B). These data highlight that pDCs are the immune cells that have the highest mRNA expression of IFN $\lambda$ R1 in tumors, even though NK cells, proliferating T cells, and then B cells seem to have a small expression of IFN $\lambda$ R1. For IL-10R $\beta$ , TA-pDCs also express a high level of this chain but the highest expression is reached by M2 macrophages (Fig. 7B). One limit of the public transcriptomic data is that not all DC subsets are presented. Thus, we cell-sorted cDC1, cDC2, pDCs and Langerhans cells (LC) from breast tumors and perform bulk RNA sequencing to evaluate their respective expression of the IFN-III receptor chains (Fig. 7C). Within tumor-associated DC (TA-DC) subsets, pDCs remain the subset with the highest expression of IFN $\lambda$ R1 (Fig. 7C). Concerning IL-10R $\beta$ , its expression is strong in cDC2, LC, and pDC, but weak in cDC1.

To confirm that TA-pDCs IFN-III response is functional, we processed breast, ovarian, and lung tumors to obtain cell suspension of TA immune cells and stimulate them with IFN-I or IFN-III. Where all immune cells responded to IFN-I, TA-pDCs were the only immune cells to respond to IFN-III, with a significant increase of pSTAT1 (Fig. 7D-E). Altogether, we shown that in various tumor type, pDCs are the immune cells with the highest expression of IFN $\lambda$ R1 chain and respond to IFN-III with a high level of pSTAT1.

### **IFN-III overcome TGF- $\beta$ inhibition by enhancing pDC production of IFN-I upon TLR7 stimulation**

Even though TA-pDCs respond to IFN-III, it has been reported that IFN- $\alpha$  production by pDCs is inhibited by the presence of TGF- $\beta$  and TNF- $\alpha$  (Hartmann et al. 2003; Bekeredjian-Ding et al. 2009; Sisirak et al. 2013). Our team gathered 69 supernatants from primary dilacerated breast tumors (SN-Dil) and quantify the concentration of TGF- $\beta$ 1, TNF- $\alpha$  and IL-10, three cytokines with inhibitory properties. As shown in Fig. 8A the cytokine that was most likely to inhibit IFN-I production of TA-pDCs was TGF- $\beta$ 1 with SN-dil containing ~1000 pg/mL of TGF- $\beta$ 1 and no TNF- $\alpha$  and no IL-10.

By looking at normalized counts of *TGFBR1* genes, we found that IFN-III stimulated pDCs had a significant decrease of TGFBR1 expression, with a p-value = 0.029 (Fig. 8B). The normalized counts of *TGFBR2* gene remained unaffected by IFN-III treatment (data not shown). As IFN-III strongly increase IFN-I production, along with TGFBR1 decrease expression, we wondered if IFN-III could counteract TGF- $\beta$  inhibition. To answer this question, we isolated pDCs from

PBMCs, cultivated them +/- IFN-III and/or TGF- $\beta$  before adding IMQ (Fig. 8C). As described previously, TGF- $\beta$  pre-treatment does inhibit pDCs, as a decreased PD-L1 expression is observed in response to IMQ (38% in the control compared to 22% with TGF- $\beta$ ) (Fig. 8D). More interestingly, IFN-III counteract TGF- $\beta$  inhibition. Adding IFN-III with TGF- $\beta$  in pDCs pre-treatment restore PD-L1 expression as the percentage of PD-L1+ pDCs reached 55%.

Finally and importantly, by quantifying IFN- $\alpha$  in the supernatant, we observed that IFN- $\alpha$  production can be restored when pDCs are pre-treated with IFN-III. This clearly demonstrate that IFN-III can counteract the TGF- $\beta$  inhibition of pDCs (Fig. 8G).

### **cDC1 and pDCs are in close contact or in the same location in the TME**

We previously demonstrated that cDC1 were the only cells able to produce IFN-III in the TME (Hubert et al. 2020). To acquire an efficient IFN-producing phenotype, pDCs should be located close enough to cDC1 secreting IFN-III. To determine pDCs and cDC1 localization in the TME, we performed multiplex-Immunofluorescence (mIF) staining on 10 breast tumors. We first observed numerous cDC1 (XCR1+) co-localizing with pDCs (BDCA2+) (Fig. 9A). Using image analysis software (Inform Software), and bioinformatic tools, we quantified pDC and cDC1 close contact (distance from the center of two nuclei < 15 $\mu$ m). 10% of cDC1 are in close contact with one pDCs (Fig. 9B-C). However, as IFN-III are cytokines, immune cells does not have to be in close contact to benefit from its action. Hence, we assess the number of pDCs present in a 100, 70 and 30  $\mu$ m radius of a cDC1. We find that 4, 2, and 1 pDCs are present in cDC1 radius respectively (Fig. 9D). These results demonstrate that TA-pDCs are often located near a TA-cDC1 and can benefit from their IFN-III secretion.

## **DISCUSSION**

In conclusion, our results highlight the mechanisms induced by IFN-III on pDCs to increase their production of IFN-I and reinforce the possibility of using IFN-III as immunotherapy. IFN-III can restore IFN-I production by pDCs with the use of a lower dose of TLR7 agonist. Indeed, we were able to demonstrate that IFN-III increases the expression of TLR7 on pDCs, making them more sensitive to low dose of TLR7 agonist. After stimulation with IFN-III and activation with a TLR7 agonist, pDCs have a very strong increase in IFN-I secretion. Finally, we also demonstrated that IFN-III counteract TGF- $\beta$  inhibition.

We used exogenous TGF- $\beta$  to recreate the TME inhibition of pDCs. To go further, it would be interesting to expose pDCs to SN-Dil to correctly mimic TME inhibitory impact and test the effect of IFN-III supplementation. Using SN-Dil is important as TA-pDC could also be inhibited by other molecules such as lipids (i.e. PGE<sub>2</sub>), or enzymes (i.e. arginase1) and could lead to an immunosuppressive environment (Arlaukas et al. 2018; Bekeredjian-Ding et al. 2009). Moreover, the mechanism involved in IFN-III counteraction of TGF- $\beta$  remains unknown. One hypothesis is that IFN-III induces a downregulation of TGF $\beta$ R1 at the protein level inhibiting TGF- $\beta$  signaling. IFN-III could also inhibit of TGF- $\beta$  downstream signaling by an inhibitory intracellular factor. For instance, smad2 and smad3 phosphorylation might be impaired by IFN-III signaling. Another hypothesis would be that IFN-III induction of IFN-I production is so strong in pDCs that TGF- $\beta$  pathways is not sufficient to inhibit IFN-I production.

pDCs high infiltration in TME was associated with poor prognosis in breast (Treilleux et al. 2004; Sisirak et al. 2012) and ovarian cancer (Labidi-Galy et al. 2011; 2012), but it was also associated with a good prognosis in colorectal (Kießler et al. 2021), pancreas (Plesca et al. 2022) and breast cancers (Michea et al. 2018; Oshi et al. 2020). It is therefore still difficult to determine the prognosis of pDC infiltration in tumors.

The immunostimulatory properties of IFN-III stimulated pDCs could be debated because they upregulates PD-L1 and ICOS-L. ICOS-L induces Treg expansion only in the absence of IFN-I (Faget et al. 2012). However, IFN-I attenuates Treg functions in the TME (Gangaplara et al. 2018). Therefore, even if IFN-III stimulated pDCs express ICOS-L at their surface, their own production of IFN-I may be sufficient to inhibit Treg expansion. Finally, pDCs exposed to IFN-III may secrete such a high dose of IFN-I that its impact on other immune cells could overcome immunomodulatory effects induced by PD-L1 expression. If T cell anergy induced by PD-L1 expression on IFN-III-stimulated pDCs could be overcome by combining IFN-III with immune checkpoint inhibitors. This could be good strategy to overpass PD-L1 immunosuppressive properties. In the light of these observation, it is pDC state of activation that should be taken in consideration regarding their prognosis more than their rate of infiltration.

We initially screened IFN $\lambda$ R1 expression on bulk RNA-seq from human and mice public data. In human, these databases did not include neutrophils. However, mice database included neutrophils. In mice, neutrophils are the immune cells that express the highest level of *IFN $\lambda$ R1* gene. Interestingly, pDCs do not express *IFN $\lambda$ R1* gene in mice. This observation can be link to the fact that no studies investigated the role of IFN-III on pDCs in mice. In public data of scRNA-seq including neutrophils, IFN $\lambda$ R expression was not detected in neutrophil cluster (data not shown). Moreover, it has been reported that human neutrophils can express *IFN $\lambda$ R1*

genes only after TLR4 stimulation (Broggi et al. 2017). Hence, at steady state, human neutrophils seem to not express IFNAR1.

We demonstrated that pDCs pre-treated with IFN-III gain the capacity to produce several cytokines and chemokines (IL-6, TNF- $\alpha$ , CX3CL1, MIP1-b). A synergic action of IFN- $\alpha$  and IL-6 was described to favor B cell differentiation into plasma cells (Jego et al. 2003). IL-3 increased the proportion of PD-L1- CD80+ pDCs secreting higher expression of CXCL10, and CCL19. CXCL10 is commonly known to increase CD8+ T cell recruitment in human malignant melanoma lesions (Kunz et al. 1999; Harlin et al. 2009; Dengel et al. 2010). CCL19 is associated with T cell recruitment and T cell activation in HBV (Yan et al. 2021). This supports the concept that IL-3 reinforce pDCs APC properties (Alculumbre et al. 2018; Ito et al. 2001).

It was demonstrated in mice that pDCs undergo three stages of maturation after viral infection (Abbas et al. 2020). pDCs start by producing IFN-I and continue their maturation until they acquire functional antigen presentation capacities. Nevertheless, evidence of this pDCs trajectory has yet to be demonstrated in human. In our experiment, we identified three subpopulations of pDCs across different stimulations, but it would have been interesting to analyze these pDCs throughout the course of activation and on a longer period of time. Indeed, IL-3 stimulated pDCs could have reach their final state of maturation more rapidly than IFN-III stimulated pDCs. We showed that after 42h of culture, a high quantity of IFN-I (8 ng/mL) was present in IL-3 stimulated cells even though only 15% of pDCs had a P1 subpopulation phenotype and 43% a P3 phenotype. This IFN-I production might be due to the small fraction of IFN-I producing pDCs. But it may also be the reminiscence of mature pDCs which went through a stage of IFN-I production during their maturation process.

Finally, to further investigate the potential of IFN-III as immunotherapy, investigation *in vivo* shall be performed. The use of *IFNAR1*<sup>-/-</sup> deficient mice could provide key information on IFN-III contribution in tumor growth. Moreover, intratumor injection of TLR7 combined with IFN-III could also be performed in tumor bearing mice. This strategy would allow to use lower doses of TLR7 agonists and then potentially limit classical associated severe adverse events in human.

## **Material and Methods:**

### ***pSTAT1 staining :***

Cells suspension were treated with 100 ng/mL IFN-L1/IL-29 (Peprotech – REF: #300-02L) or 1,000 U/mL IFN- $\beta$  (Peprotech – REF: #300-02BC) for 45min at 37°C. Cells were then stained with zombie NIR (1/400, ref : 423106, Biolegend) during 20 min at 4° and antibody mix containing : anti-BDCA2 (clone : L307.4, BD, diluted 1/50), anti-CD123 (clone : 7G3, BD, diluted :1/100), anti-HLA-DR (clone : L243, Biolegend, diluted :1/50), anti-CD3 (clone :UCHT1, BD, diluted : 1/50), anti-CD20 (clone : 2H7, BD, diluted 1/20), anti-CD14 (clone : A59, BD, diluted : 1/50). BD Lyse/Fix Buffer (BD 558049) was used to fix cells during 10 min at 37°C 5% CO<sub>2</sub>. This step was followed by a cell permeabilization with Perm Buffer III (BD 558050) during 30 min on ice. 50  $\mu$ L/well of SVF were added before washing with FACS twice. Pellets were resuspended in antibody mix containing : anti-pSTAT1 (ref : 612597, clone :4a, BD, diluted :1/10), anti-CD11c (clone : Bu15, Biolegend, diluted :1/20), anti-CD56 (clone :901, Beckman Coulter, diluted :1/50) and anti-CD45 (clone : HI30, BD, diluted :1/25) and incubated in the dark (RT) for 40min.

### ***Analysis of public transcriptomic data :***

IL-28R $\alpha$  or IL-10R $\beta$  RNA normalized counts were retrieved from the Human Cell Atlas (HCA) to analyze their expression in PBMCs, or were retrieve from the Tumor Immune Single-cell Hub (TISCH) to analyze their expression in tumor-associated immune cells. The bar plot graph were created with R studio software.

### ***Plasmacytoid dendritic cells isolation :***

From 500ml healthy donors blood, lymphocyte isolation using density gradient centrifugation was done thanks to Lymphocyte separation medium (Eurobio scientific – REF: ). We then performed a first myeloid cell enrichment by using pouring 3ml of PBMCs at à 0,333.108 cells/mL on 6 ml of Percoll solution (GE Healthcare – REF: ). After 20min of centrifugation at 2000 rpm (without brakes or acceleration) the myeloid layer was harvested. Plasmacytoid dendritic cells isolation was then performed with Plasmacytoid Dendritic cells isolation Kit II, human (Milteni, Ref : 130-092-207).

### ***Cells Viability and phenotype characterization:***

pDCs isolated from PBMCs were always placed at a concentration of 1.10<sup>6</sup> cells/ml in round shaped 96-well plates in a final volume of 200  $\mu$ L. pDCs were treated with or without 100 ng/ml IFN-L1/IL-29 (Peprotech – REF: #300-02L) and/or 20 ng/ml IL-3 (Peprotech - REF : 200-03) for 24h at 37°C 5% CO<sub>2</sub>. After stimulation, pDCs were stained 15 min at 4°C with zombie NIR

(1/400, ref : 423106, Biolegend), followed by an antibody staining of 30 min at 4°C containing: anti-CD123 (ref: 563405, clone:7G3, BD, diluted 1/100), anti-BDCA2 (ref : 1294661, clone : V24-785, BD, diluted 1/50), anti-PD-L1 (ref : B285480, clone : 29E2A3, Biolegend, diluted :1/50), anti-ICOS-L (ref : 2017863, clone : MIH12, Invitrogen, diluted 1/50), anti-HLA-DR (ref :B314704, clone : L243, Biolegend, diluted 1/50), anti-CD80 (ref : 7046635, clone :L307.4, BD, diluted : 1/50), anti-CD86 (ref : 93222850, clone : 23331(FUN-1), BD, diluted 1/50). Cells were fixed at RT for 45 min in the dark with of FA4% (Sigma-Aldrich – REF: F8775-500ML). All flow cytometry acquisitions were done on an LSRFortessa Cell Analyzer (BD Biosciences), and data were processed in FlowJo 10.4 (Tristar).

#### ***Quantitative real Time RT-PCR:***

pDCs were incubated for 6h, 12h or 18h with IL-29 (100 ng/mL) at 37°C 5% CO<sub>2</sub>. pDCs were then lysed with a 1% 2-β-Mercaptoethanol (Sigma-Aldrich, M6250-100ML) + TCL Buffer (Qiagen, 1031576) solution. Total RNAs were then extracted from cultured cells using single cell RNA purification kit (NORGEN, ref : 51800) according to the manufacturer's instruction. RNA retro-transcriptoin into cDNA was performed with I Script cDNA Synthesis Kit (BioRad, ref: #172-5038). SsoAdvanced universal SYBR Green super Mix was used for quantitative polymerase chain reaction in real-time. Probes sequences were design in house, with OAS2 Fw : "5'-AGGGAGTGGCCATAGGTGG-3'", OAS2 Rv : "5'-AACACCTGGATGGTGAACCC-3'", GAPDH Fw: Rv:. Probes were manufactured by .qPCR was performed with CFX Real-Time PCR system machine (BioRad), with the following program: 1 min hold at 95°C, 39 cycle of 5 seconds at 90°C (denaturation) and 10 seconds at 60°C (annealing/extension). Each sample was analyzed in duplicate, and the experiments were done twice. A non-template control (RNA-free water) was included on every plate.

#### ***RNA Sequencing and data analysis of purified pDCs:***

RNA sequencing was performed on untreated or IFN-III treated (100ng/ml) isolated pDCs for 12h at 37°C. pDCs were then lysed with a 1% 2-β-Mercaptoethanol (Sigma-Aldrich, M6250-100ML) + TCL Buffer (Qiagen, 1031576) solution. Total RNAs were then extracted from cultured cells using single cell RNA purification kit (NORGEN, ref : 51800) according to the manufacturer's instruction. The sequencing and QC analysis were then carried out by the Cancer Genomics Platform of the Leon Berard Center. Data normalization was then done with the DEseq2 R packages. PCA and volcano plot were performed with DEseq2 R packages. DEG between pDCs treated or not with IFN-III were defined with a  $|\log(\text{Foldchange})| > 0.58$  and adjusted p-value  $< 0.05$  and z-scores computed with R studio. Z-score heatmap of of the complete list of DEG was then uploaded on Morpheus website created by the Broad Institute. GSEA analysis was performed from the GSEA-MSIGB software using

the « h.all.v7.5.1.symbols.gmt » gene set. Venn diagram was created with ggvenn R package on the enriched genes from “Interferon gamma response” and “Interferon alpha response”. Normalized counts from specific gene of interest were retrieved from the normalized count table created by DEseq2 and plotted using ggplot2 R package.

***TLR7 and TLR9 flow cytometry staining :***

pDCs isolated from PBMCs were treated with or without 100 ng/ml IL-29 for 18h at 37°C 5% CO<sub>2</sub>. Cells were the stained 15min at 4°C with zombie NIR and incubated 30min at 4°C with an antibody mix containing anti-CD123 and anti-BDCA2. Fixaxtion/permeabilisation step were performed with concentrate solution with FOXP3 /transcription factor staining buffer set, (Invitrogen , lot :2171417 - ref :00-5523-00) for 20min at 4°C before to be permeabilized with permeabilization buffer (Invitrogen, ref :00-5523-00). pDCs were then stain with anti-TLR7 (R&D, ref : IC5875P, clone : 533707, diluted :1/100) or anti-TLR9 (Ebioscience, ref: 12-9099-82, clone: eB72-1665) during 30min at 4°C. All flow cytometry acquisitions were done on an LSRFortessa Cell Analyzer (BD Biosciences), and data were processed in FlowJo 10.4 (Tristar).

***Imiquimod activation:***

To asses IMQ suboptimal dose, pDCs isolated from PBMCs were treated with 100 ng/ml IL-29 and or IL-3 (20 ng/mL) for 18h at 37°C 5% CO<sub>2</sub>, and different concentration of IMQ were add to pDCs culture for 24h, and cells were then stained for flow cytometry analysis and phenotyping. pDCs were treated with 100 ng/ml IL-29 for 18h at 37°C 5% CO<sub>2</sub>, before to be activated with the strong dose (250 ng/mL) or the lower dose of IMQ (50 ng/mL). No pretreated pDCs were kept at 4°C for 18h before IMQ activation. After 42h of incubation cells were harvested and stained for membrane markers.

***Tumors dilaceration and tumor cell suspensions :***

Fresh tumors were obtained from the Biological Resources Center (BRC) of the CLB (Lyon, France) and from the TUMOROTHEQUE (BRC of the Hospices Civils de Lyon, France). Received tumors are weighed and arranged in a petri dish with RPMI 0% SVF per 500g of tumors. Tumors were then manually dilacerated and SN-dil were harvested. Enzymatic digestion was performed with DNase I (Sigma, ref: D4513) and 200UI/ml Collagenase IV (Gibo, ref: 17104-019) in 10ml RPMIc 20% SVF for 45min at 37°C. The whole solution was filtered on 70µM strainer. Single cell suspension were then obtain and used or pSTAT1 staining.

### ***Sorting of TA-DC subsets***

Single-cell suspensions were stained using antibodies listed in table 1. Dying cells were excluded by DAPI staining. Lymphocytes, NK cells, neutrophils, and other myeloid cells (monocytes, macrophages, and inflammatory monocytes) were also excluded using respectively anti-CD3/56/15/14 antibodies in the lineage. In the HLA-DR<sup>+</sup> Lin<sup>-</sup> gate, DC subsets were identified using the following phenotypes: CD11c<sup>-</sup>/low CD123<sup>+</sup> pDC, CD11c<sup>+</sup> BDCA1<sup>-</sup> BDCA3<sup>high</sup> CLEC9A<sup>+</sup> cDC1, CD11c<sup>+</sup> BDCA1<sup>+</sup> BDCA3<sup>-</sup> CD207<sup>-</sup> cDC2 and CD11c<sup>+</sup> BDCA1<sup>+</sup> BDCA3<sup>-</sup> CD207<sup>high</sup> LC. Using a BD FACSAria III, 1000 cells of each identified TA-DC subset were then sorted in TCL buffer (Qiagen) supplemented with 1%  $\beta$ -mercaptoethanol (SIGMA) for RNA-seq experiments.

### ***RNA-seq analysis and data pre-processing of TA-DC subsets***

RNA from sorted samples was extracted by using a Single Cell RNA Purification Kit (Norgen Bioteck), including on-column DNase digestion (Qiagen), as described by the manufacturer's protocol. cDNA was generated with SmartSeqV4 oligodT primer (Takara), following manufacturer's protocol. The quantity and quality of cDNA were assessed using Qubit dsRNA high sensitivity (Thermofisher) and DNA HS D5000 (Agilent) respectively. Multiplexed pair-end libraries 50nt in length were obtained using Nextera XT kit (Illumina). Sequencing was performed in the same batch in Illumina NovaSeq 6000 using an average depth of 32 million reads. Library, sequencing and quality control of the sequencing were performed by the genomic platform and the Gilles Thomas bioinformatics platform of the CLB. Reads were mapped to the human genome reference (hg19/GRCh38) using STAR software. Finally, the Salmon tool was used to quantified gene expression as read counts.

### ***Inhibition of pDCs IFN-I production by TGF- $\beta$ :***

pDCs isolated from PBMCs were treated with 100 ng/ml IL-29 and/or 2 ng/mL of TGF- $\beta$  (R&D bioscience, ref: #240-B) for 24h at 37°C 5% CO<sub>2</sub>. pDCs were activated with 50 or 250 ng/mL of IMQ for 24h. To ensure that pDCs viability was maintain thought the protocol, 5ng/mL of IL-3 was add to every condition. Supernantants were harvest after culture and kept at -80°C before to do electroluminescence assay to dose different cytokines. Cells were stained 15 min at 4°C with zombie NIR (1/400, ref : 423106, Biolegend), followed by an antibody staining of 30 min at 4°C containing: anti-CD123 (ref: 563405, clone:7G3, BD, diluted 1/100), anti-BDCA2 (ref : 1294661, clone : V24-785, BD, diluted 1/50), anti-PD-L1 (ref : B285480, clone : 29E2A3, Biolegend, diluted :1/50), anti-ICOS-L (ref : 2017863, clone : MIH12, Invitrogen, diluted 1/50), anti-HLA-DR (ref :B314704, clone : L243, Biolegend, diluted 1/50), anti-CD80 (ref : 7046635,

clone :L307.4, BD, diluted : 1/50), anti-CD86 (ref : 93222850, clone : 23331(FUN-1), BD, diluted 1/50). All flow cytometry acquisitions were done on an LSRFortessa Cell Analyzer (BD Biosciences), and data were processed in FlowJo 10.4 (Tristar).

***Distance calculation between pDCs and cDC1:***

After image acquisition, tissue and cell segmentation was performed with Inform Software. We used the machine learning module to develop an efficient algorithm for cell phenotyping able to recognize different DC subsets. Cell coordinates were then retrieved thanks to inform data frame and 2D projection were made with R studio. A pipeline was developed to calculate cell distance from one another.

***Statistics :***

Statistical analyses were performed using Prism Version 9.0 (GraphPad Software) for Friedmann test, or Excel stat for Kruscal-Wallis test. All statistical analysis were made to compare 3 groups or more with paired values corresponding to each blood/tumors donors. Thus, Friedman no parametric tests was used. Statistical significance : \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001. Each graphical representation reflects the associated non-parametric statistical test. No statistical analysis was performed when number of samples was  $\leq$  to 3.

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## **Author contributions**

C.S designed and performed experiments, did bioinformatics, analyzed results, did statistical analysis, and wrote the manuscript. A.S and C.R designed and performed experiments, analyzed results and did statistical analysis. M.H. provided the RNA sequencing and GSEA pipeline that was used and modified to fit our samples and analysis. M.A. performed the alignment and the QC of our RNA sequencing data. J.B. and B.D. set up a pipeline for the quantification of in situ stainings, A.C.D who performed all the mIF staining and E.G who developed the machine learning algorithm with Inform Software. M.H., L.L provided strategic advice and revised the manuscript. J.V-G. designed experiments, supervised the research, and wrote the manuscript.

## **Competing interests**

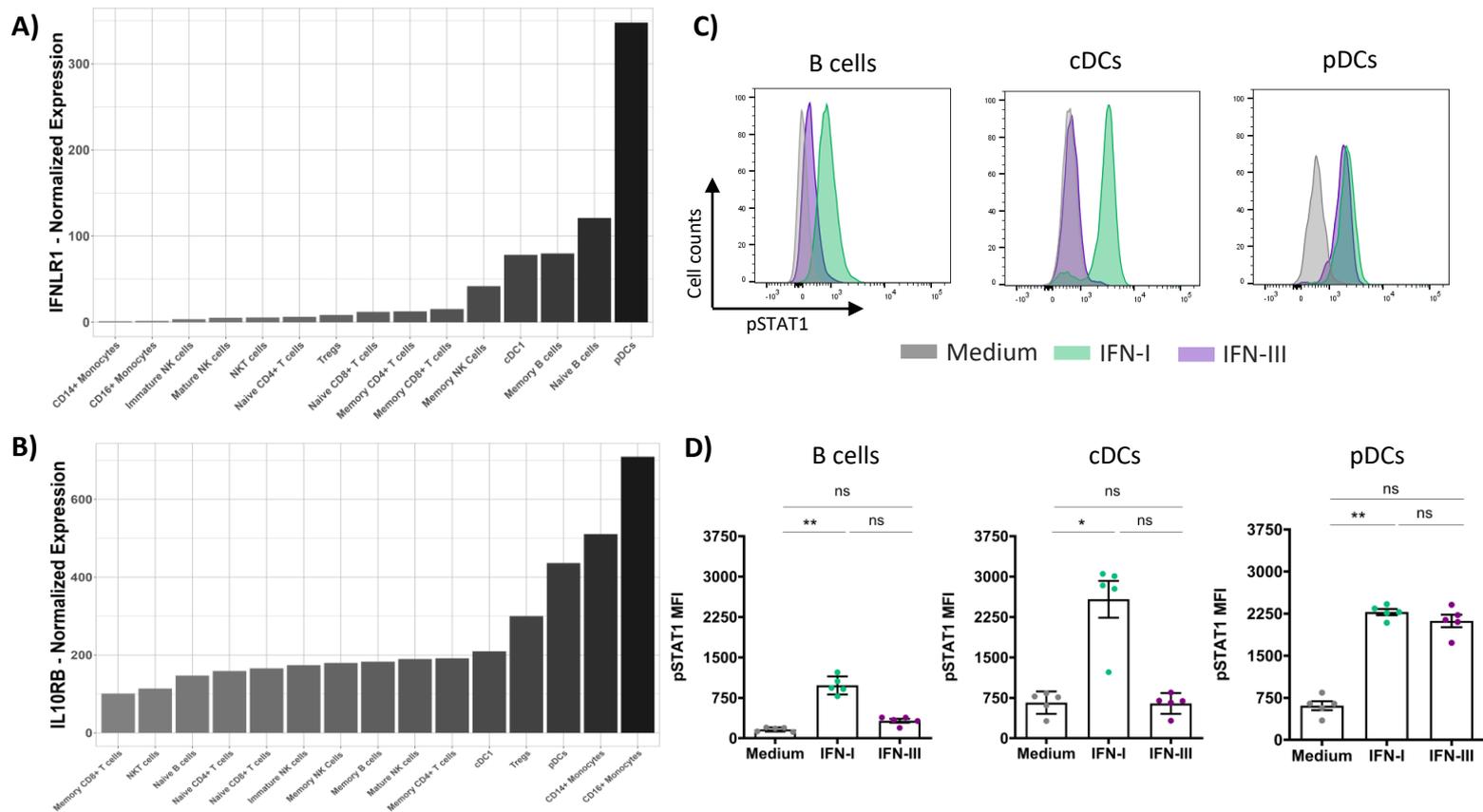
The authors declare no competing interests.

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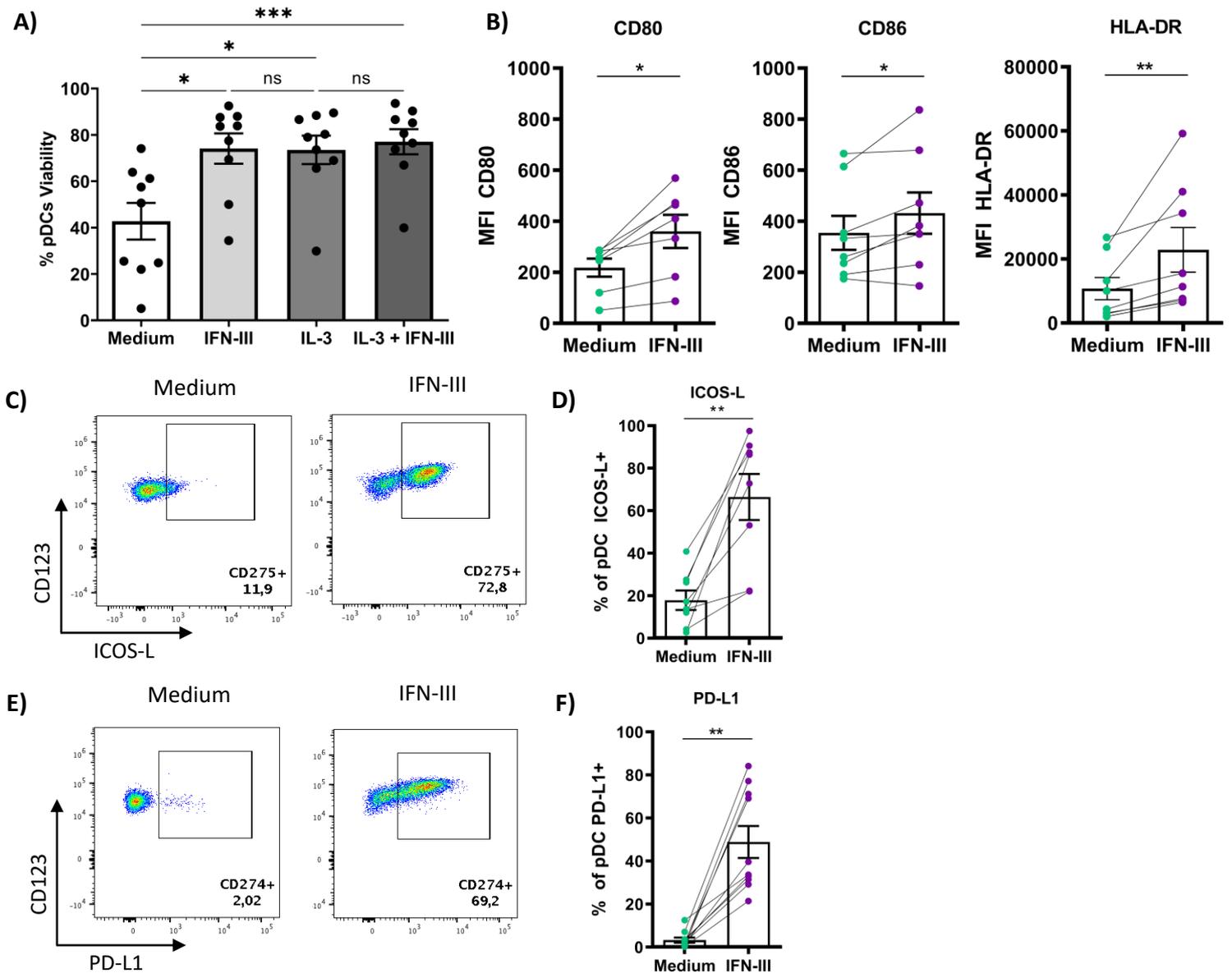
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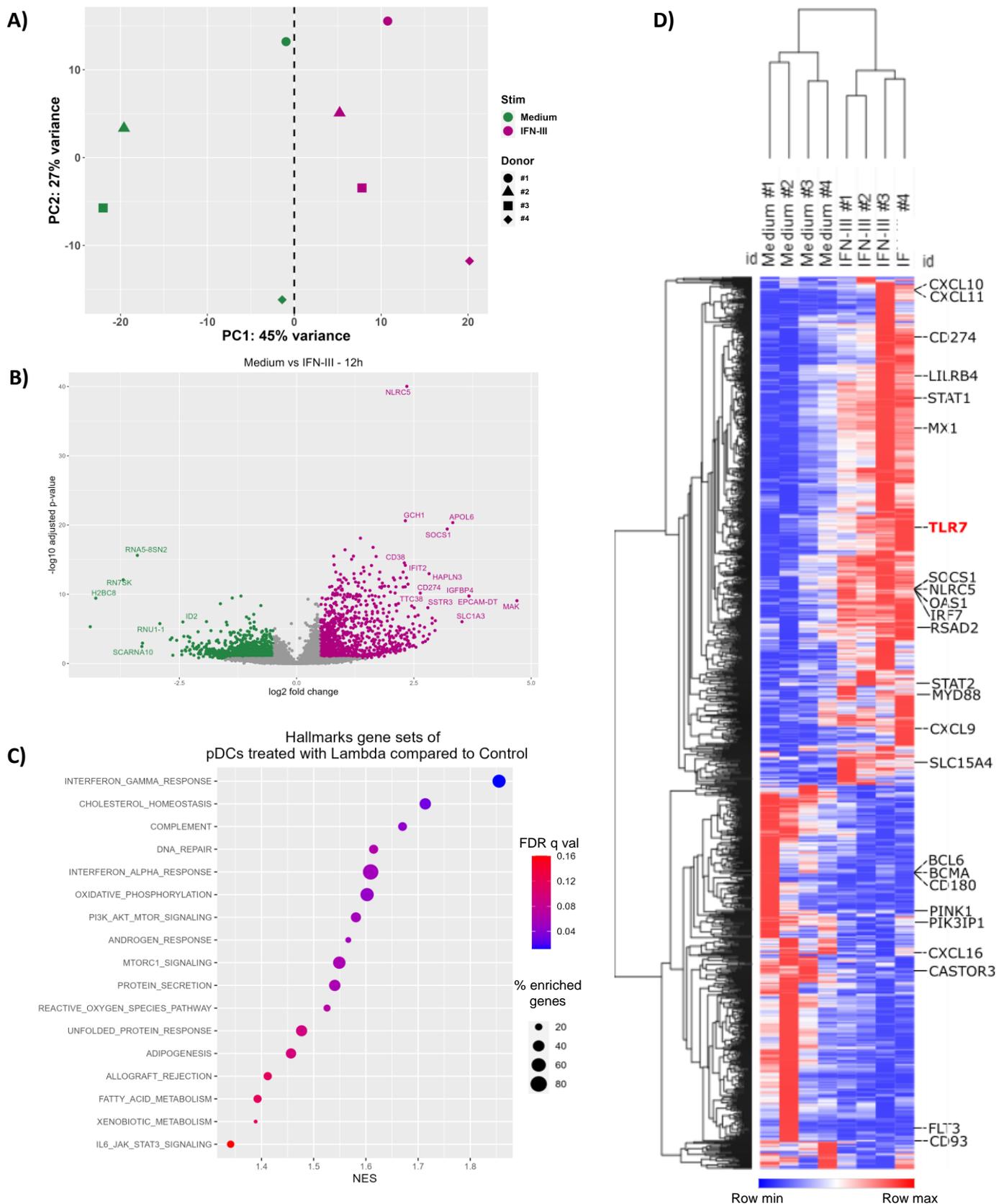


**Figure 1. pDCs strongly respond to IFN-III.**

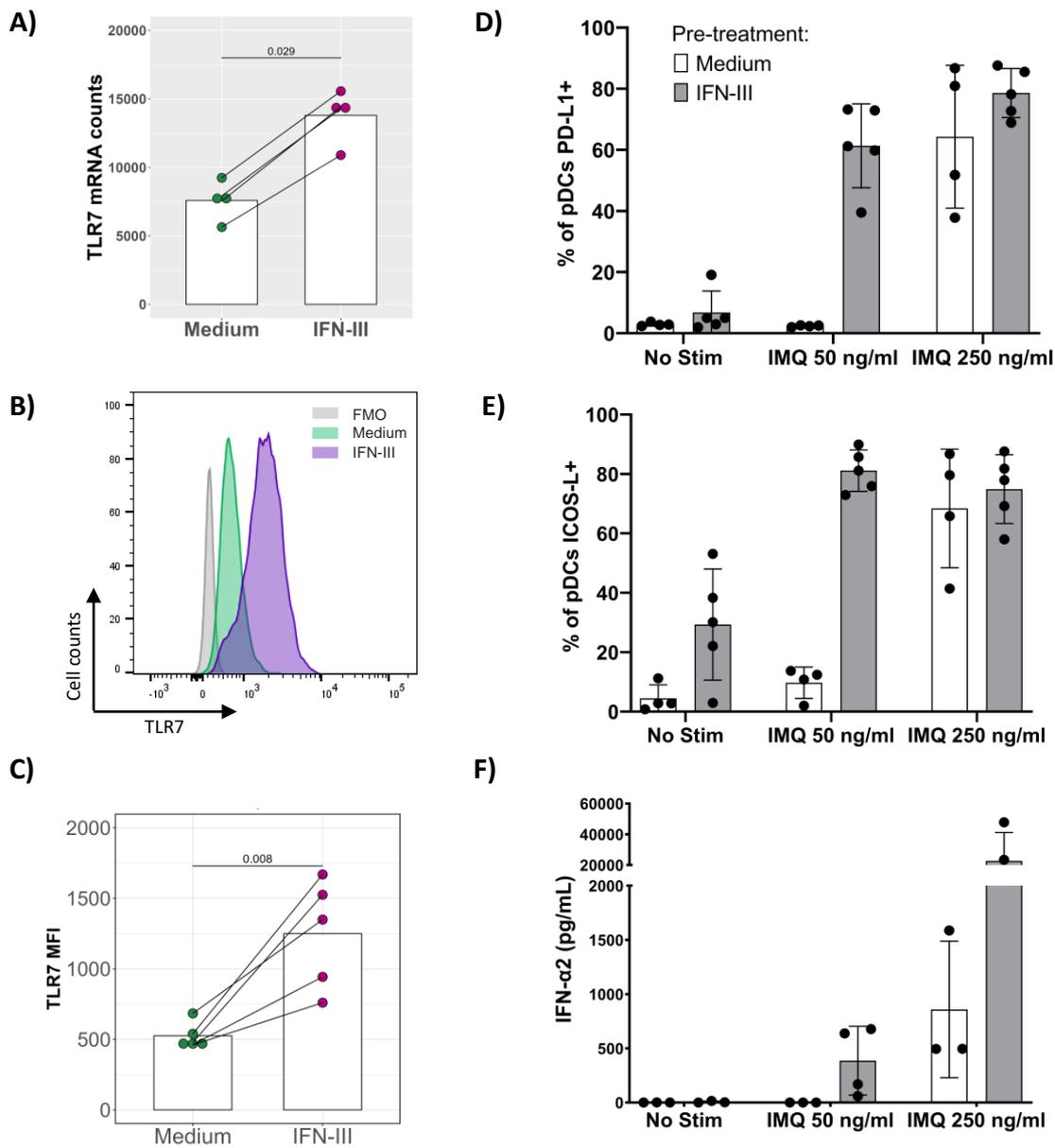
**A&B)** Normalized counts were retrieved from public data available on the Human Cell Atlas (HCA) to assess receptor expression of healthy PBMCs. **A)** IFNLR1 expression or **B)** IL10R $\beta$  expression. **C&D)** B cells, cDC and pDC pSTAT1 was measured by flow cytometry after 45min of stimulation with 100 ng/mL IL-29 or 1,000 U/mL IFN- $\beta$  (n = 5). **C)** pSTAT1 of a representative donor. **D)** pSTAT1 from healthy donors (n=5). Statistical significance was determined with Friedman tests on the MFI values: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 2. IFN-III increase activation marker expression on pDCs, along with PD-L1 and ICOS-L expression. A)** pDC survival was assessed after 24 hours treatment with or without 100 ng/mL of IL-29 and/or 5 ug/mL of IL-3. Cells were then stained with viability dye and analysed by flow cytometry (n=8). Statistical significance was determined with Friedman tests on the percentage of viability : \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. **B-E)** pDC were treated 24 hours with or without 100 ng/mL of IL-29. Cells were stained and protein expression were assessed by flow cytometry (n=8). **B)** Quantification of the MFI of activation markers CD86, CD80, HLA-DR, CD123. **C)** pDCs expressing ICOS-L from a representative healthy donor. **D)** Percentage of pDCs expressing ICOS-L at their surface (n=8). **E)** pDCs expressing PD-L1 from a representative healthy donor. **F)** Percentage of pDCs expressing PD-L1 at their surface (n=10). Statistical significance was determined with Wilcoxon tests : \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

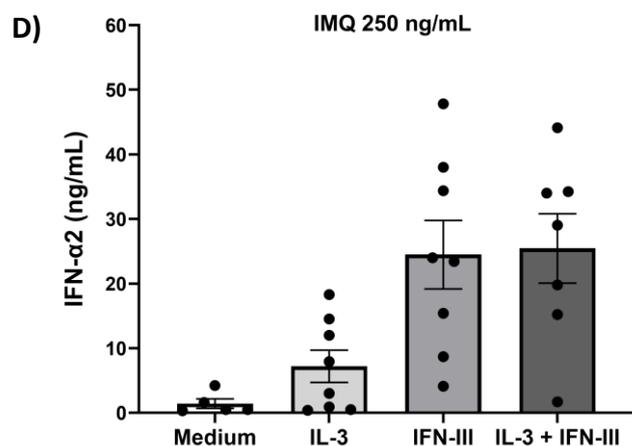
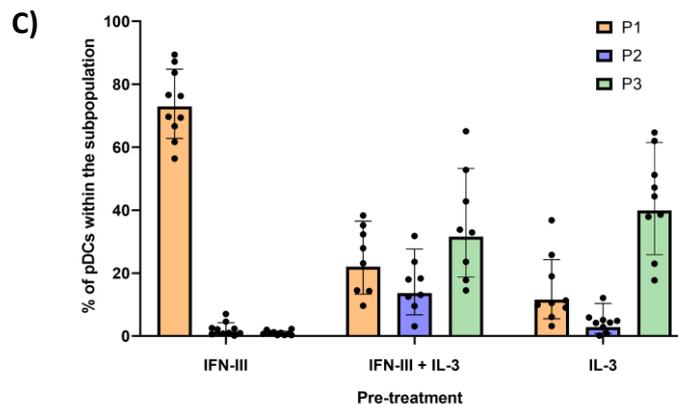
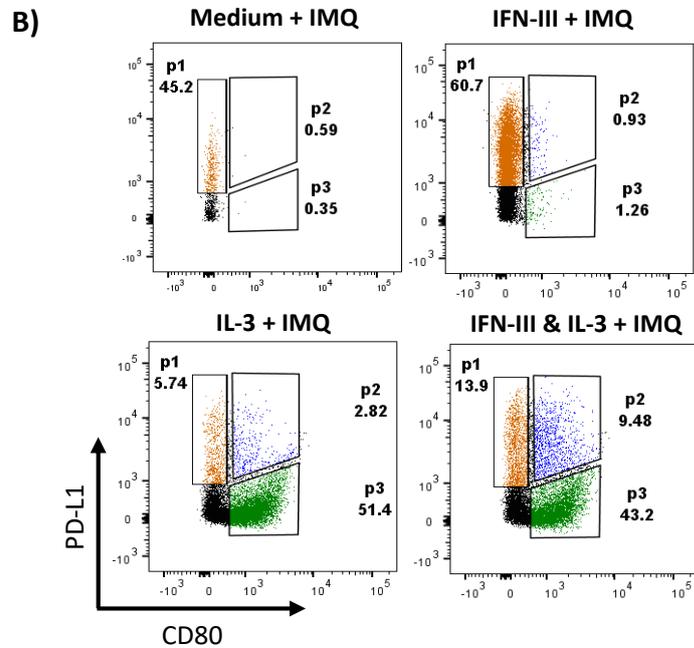
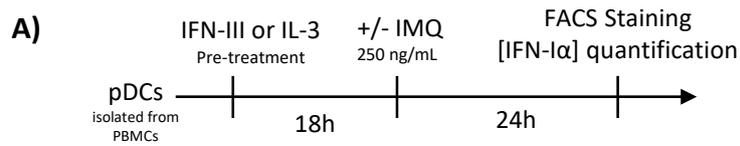


**Figure 3. IFN-III induces TLR7 and pathways involved in IFN-I production.** RNA sequencing was performed on purified pDCs treated with or without 100 ng/mL of IFN-III after 12h at 37°C. **A)** PCA of the normalized RNAseq transcripts per million (TPM) of purified pDCs treated or not with IFN-III. **B)** Volcano plot representation of differential expression analysis of genes in IFN-III treated-pDCs versus non treated pDCs. The x-axis shows log<sub>2</sub> fold changes in expression and the y-axis the -log<sub>10</sub> adjusted p-value of each genes being differentially expressed. **C)** GSEA Hallmark analysis showing enriched gene sets. The x-axis represent NES values indicating the hallmark enrichment in IFN-III treated-pDCs versus non treated pDCs. FDR q values are represented in a gradient of blue-to-red and the size of each dot represent the percentage of genes enriched for every hallmark . **D)** Heatmap of z-scores RNA-Seq expression using all 3129 DEGs. Each column corresponds to a sample and each row corresponds to a specific gene.



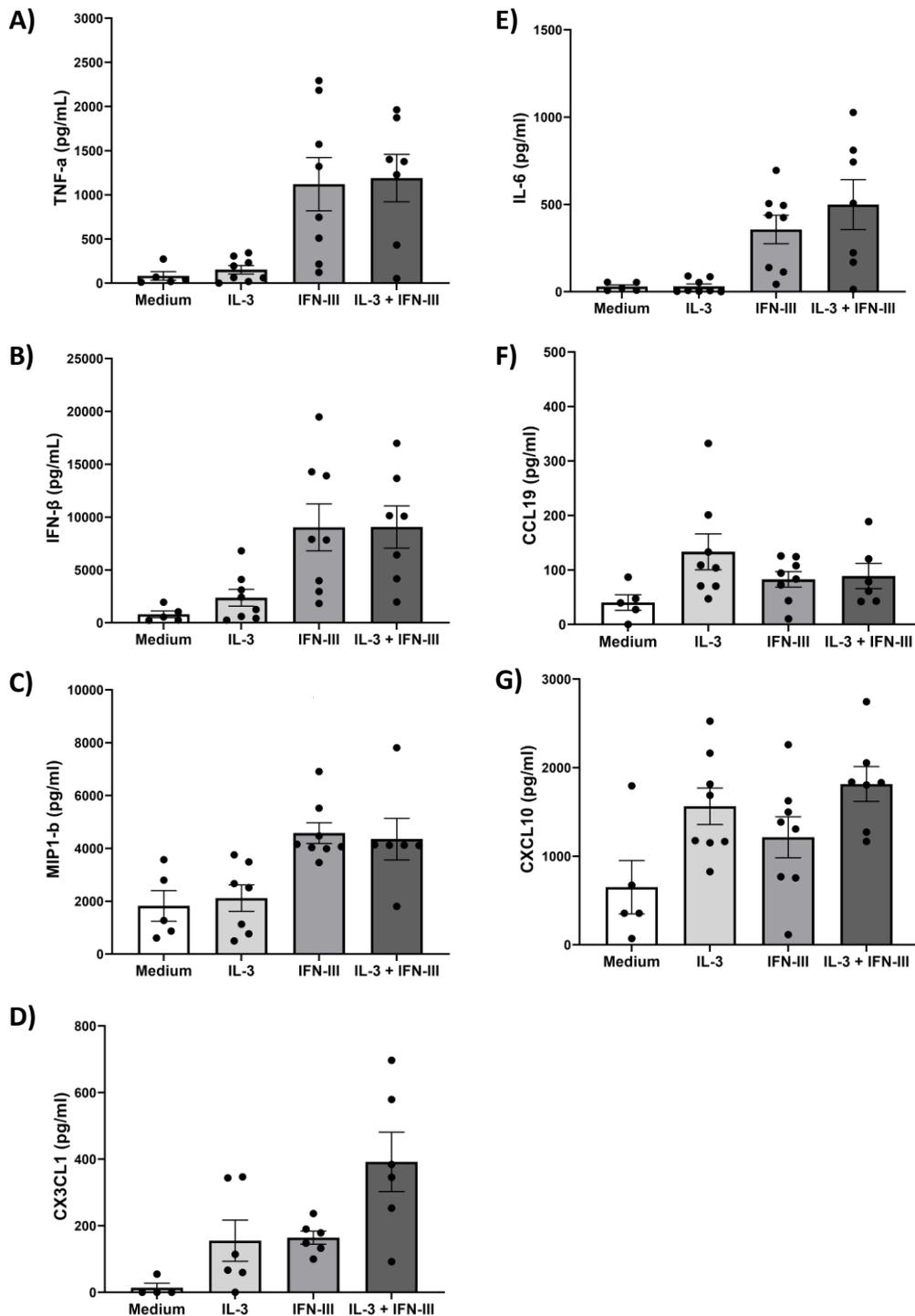
**Figure 4. IFN-III pre-treatment sensitizes pDCs to low dose of Imiquimod**

**A)** TLR7 normalized counts from RNA sequencing of pDCs treated with or without 100 ng/mL of IL-29 for 12h. Statistical significance was addressed with a Wilcoxon test. **B & C)** TLR7 protein expression was evaluated by flow cytometry purified pDCs treated with or without 100 ng/mL of IL-29 for 18h. **B)** TLR7 expression representative histogram experiment with FMO **C)** Bar plot of TLR7 expression from healthy donors ( $n=5$ ). **D)** PD-L1 and **E)** ICOS-L positive pDCs were quantified by flow cytometry after different combination of treatments with or without IL-29 (100 ng/mL) for 18h followed by 250 ng/mL or 50 ng/mL of IMQ for 24h. **F)** IFN- $\alpha$ 2a was quantified by multiplex assay in the supernatants of pDCs cultures after different combination of pre-treatments for 18h and 250 ng/mL or 50 ng/mL of imiquimod for 24h.



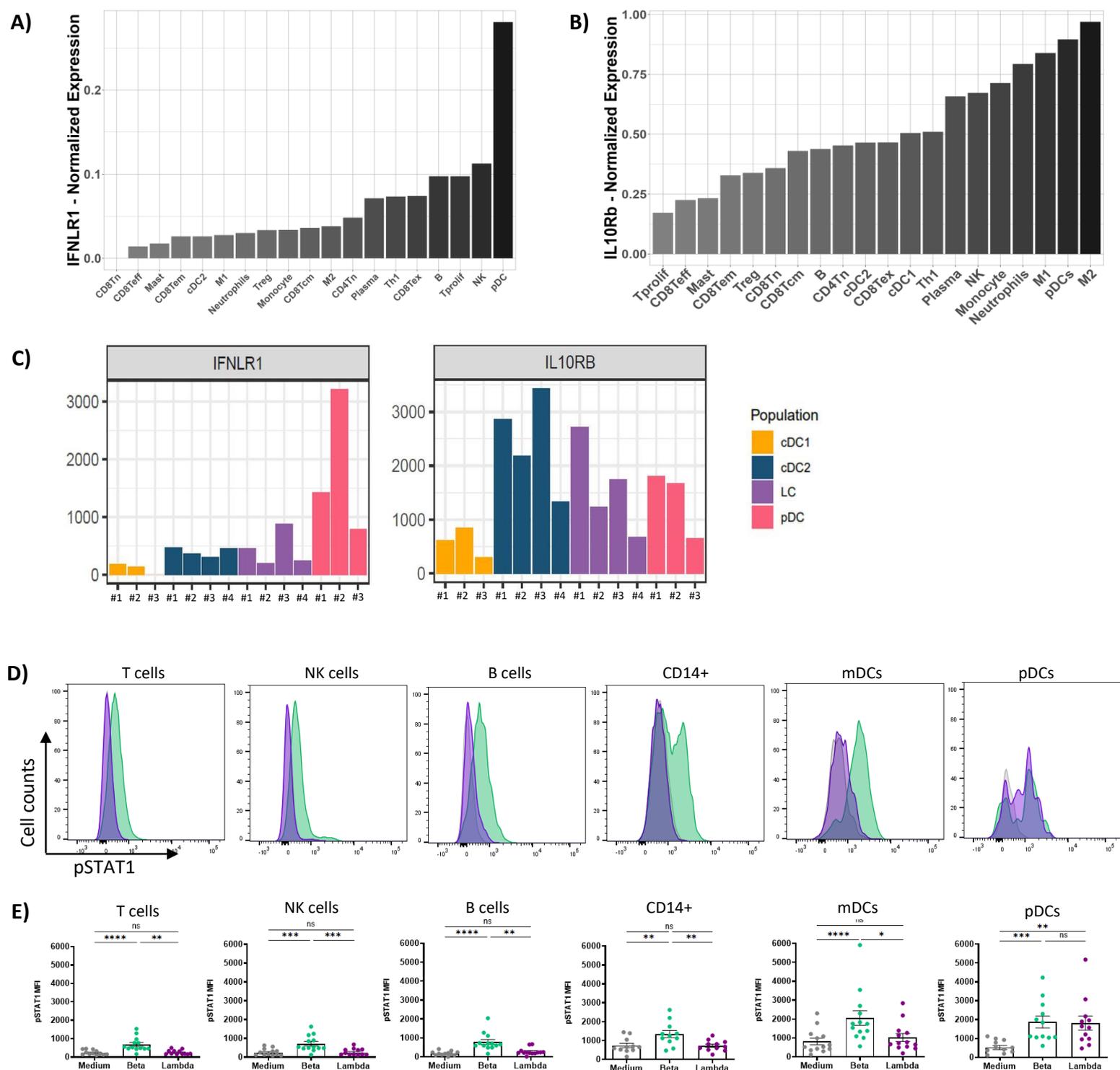
**Figure 5. IFN-III stimulated pDCs acquire an IFN-I secretion phenotype upon TLR-7 stimulation.**

**A)** Scheme representing the experiment protocol **B-C)** Percentage analysis of pDC subpopulation P1, P2, and P3 after pre-treatment and IMQ exposure. pDCs subpopulations were assessed by flow cytometry according to their expression of PD-L1 and CD80. **B)** Dot plot of representative experiment **C)** P1, P2, and P3 repartition in different pre-treatment and IMQ activation (n=8). **D)** IFN-α2a was quantified by multiplex assay in the supernatants of pDCs pre-treated with IFN-III or IL-3 for 18h before being stimulated 24h with 250 ng/mL of IMQ (n=7).

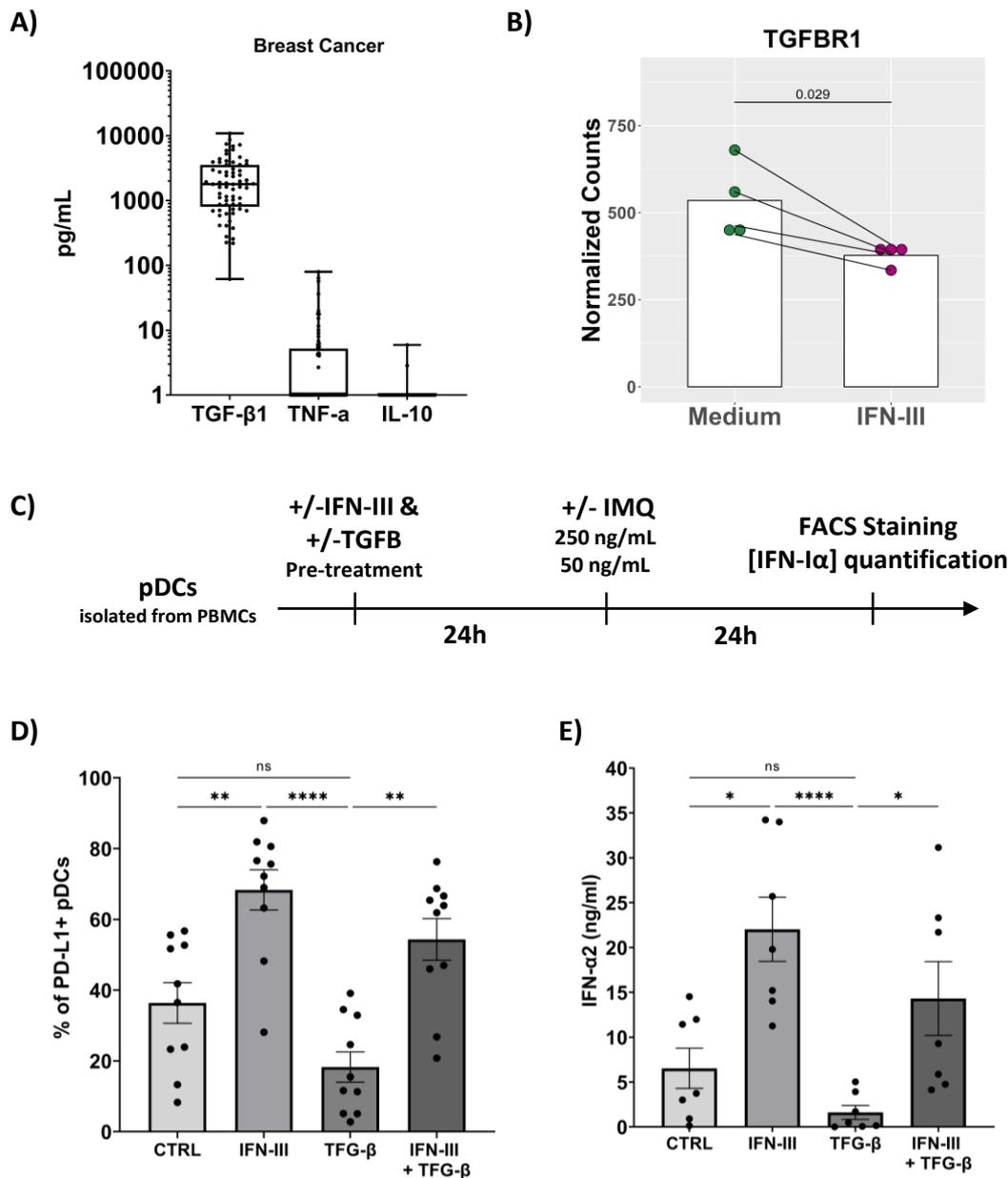


**Figure 6. IFN-III favour the secretion numerous cytokines and chemokines acting on innate immune cells**

Purified pDCs from PBMCs were pre-treated IL-29 or IL-3 for 18h before being stimulated 24h with 250 ng/mL of IMQ. Supernatants were then harvested and cytokines were quantified with multiplex assay. **A)** TNF- $\alpha$  **B)** IFN- $\beta$  **C)** MIP1-b **D)** CX3CL1 **E)** CCL19 **F)** IL-6 **G)** CXCL10.



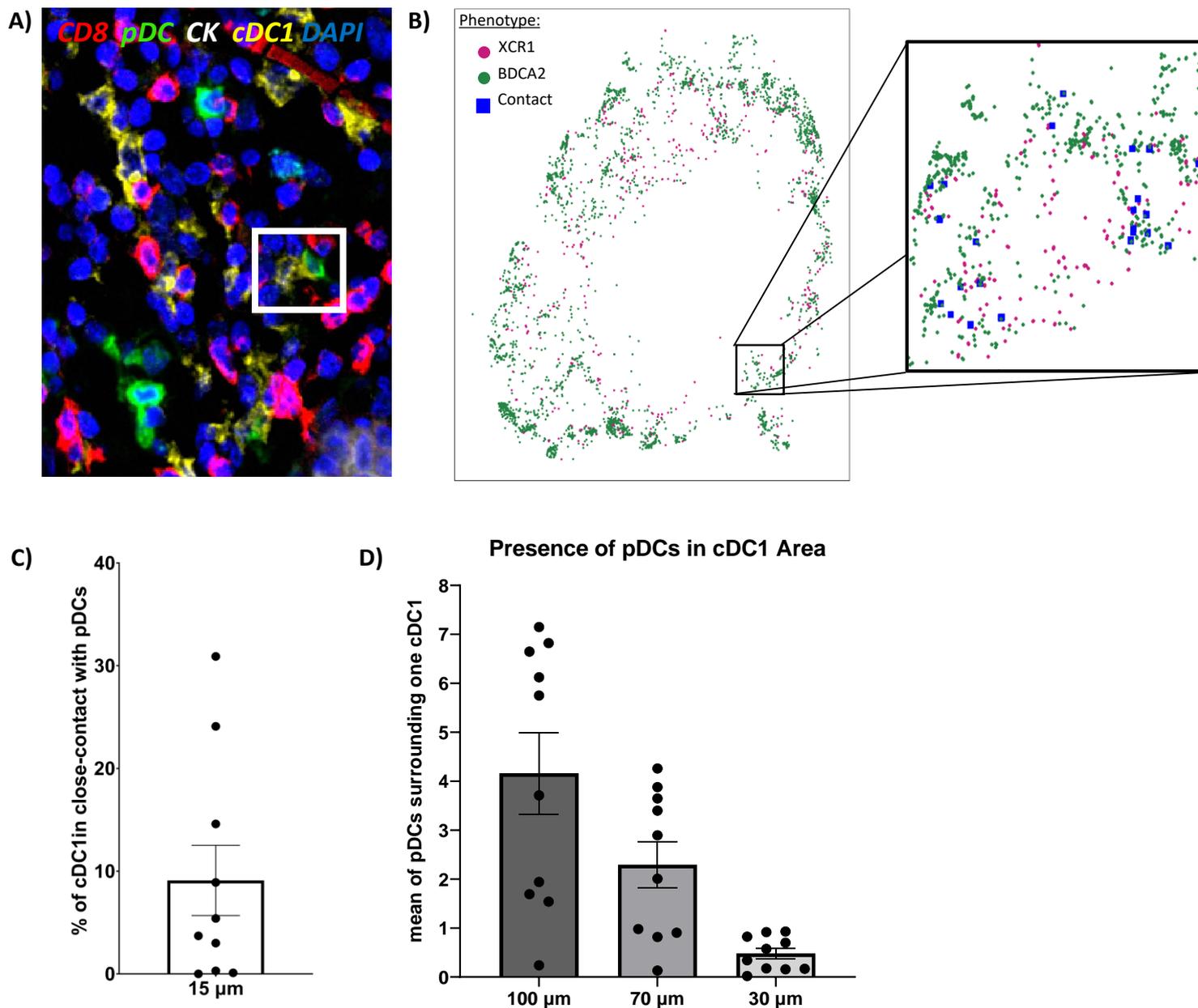
**Figure 7. Tumor-associated pDCs strongly respond to IFN-III. A&B)** Normalized counts were retrieved from public data available on the Tumor Immune Single-cell Hub (TISCH) to assess receptor expression of human immune cells present in tumors. **A)** IFNLR1 expression or **B)** IL-10R $\beta$  expression. **C)** Tumor-associated cDC1, cDC2, LC, and pDCs were cell sorted from breast tumor, RNA was then sequenced to retrieve the normalized counts of *IL-28R $\alpha$*  and *IL-10R $\beta$*  genes. **D-E)** Tumor-associated immune cells were cultivated with IFN-I or IFN-III for 45 min and pSTAT1 was analyzed. **D)** Histogram of pSTAT1 of a representative donor. **E)** pSTAT1 from the breast tumor (n=1), ovarian tumors (n=4), lung tumors (n=7). Statistical significance was determined with Friedman tests on the MFI values: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 8. IFN-III overcome TGF-β inhibition by enhancing pDC production of IFN-I upon TLR7 stimulation**

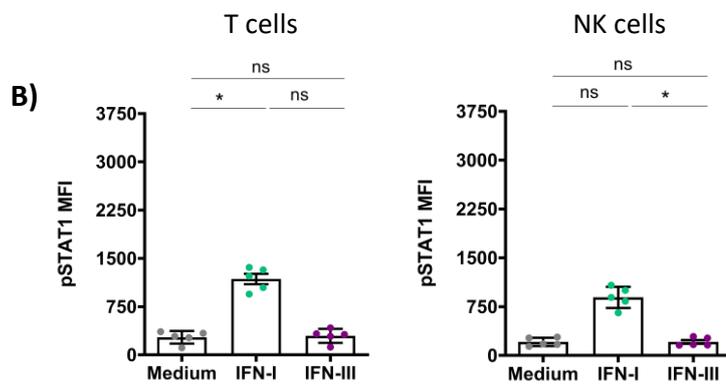
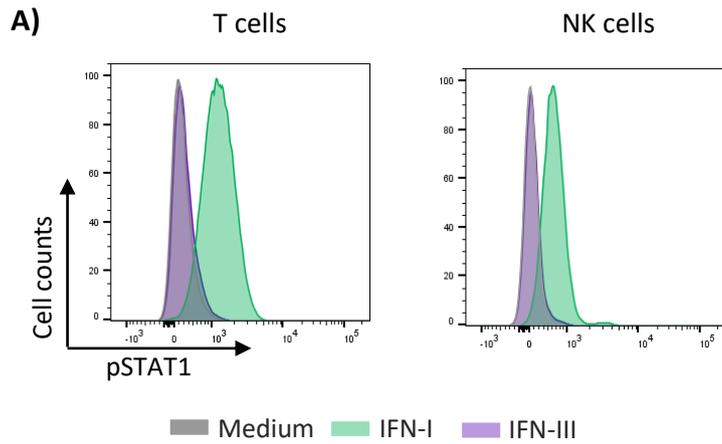
**A)** TGF-β, TNF-α and IL-10 concentrations were quantified with multiplex immune assay kit in 69 supernatants of primary dilacerated tumors (SN-Dil). **C)** Scheme representing the experiment protocol. **D)** Bar plot representing PD-L1+ pDCs after 48h treatment. **E)** Bar plot representing IFN-α2 (ng/mL) secreted by pDCs in different conditions. Statistical significance was determined with Friedman tests on the percentage of viability : \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

## Fig 8. cDC1 could induce fully functioning pDCs in the TME



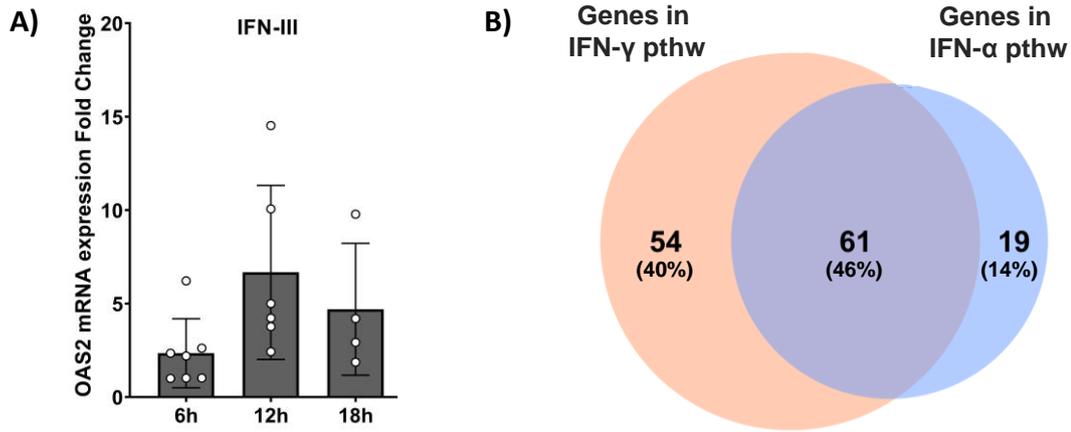
## Figure 9. cDC1 could induce fully functioning pDCs in the TME

**A)** Visualization of CD8<sup>+</sup> T cells, pDCs, cancer cells, cDC1, respectively by CD8 (red), BDCA2 (green), cytokeratin (white), XCR1 (yellow) using mIF. Nuclei were stained with DAPI. The white square focused on a close contact between cDC1 and cDC2 **B)** 2D projection of cDC1 (purple) and pDCs (green) present in a whole tumor slide of breast cancer. Blue squares represent a close contact between cDC1 and pDCs (distance from nuclei center < 15  $\mu$ m). **C)** Percentage of XCR1<sup>+</sup> cDC1 in close-contact with pDCs in ten breast tumors. **D)** The number of pDCs present in each cDC1 area (radius = 100, 70, 30  $\mu$ m) was calculated in the ten whole tumor slides.



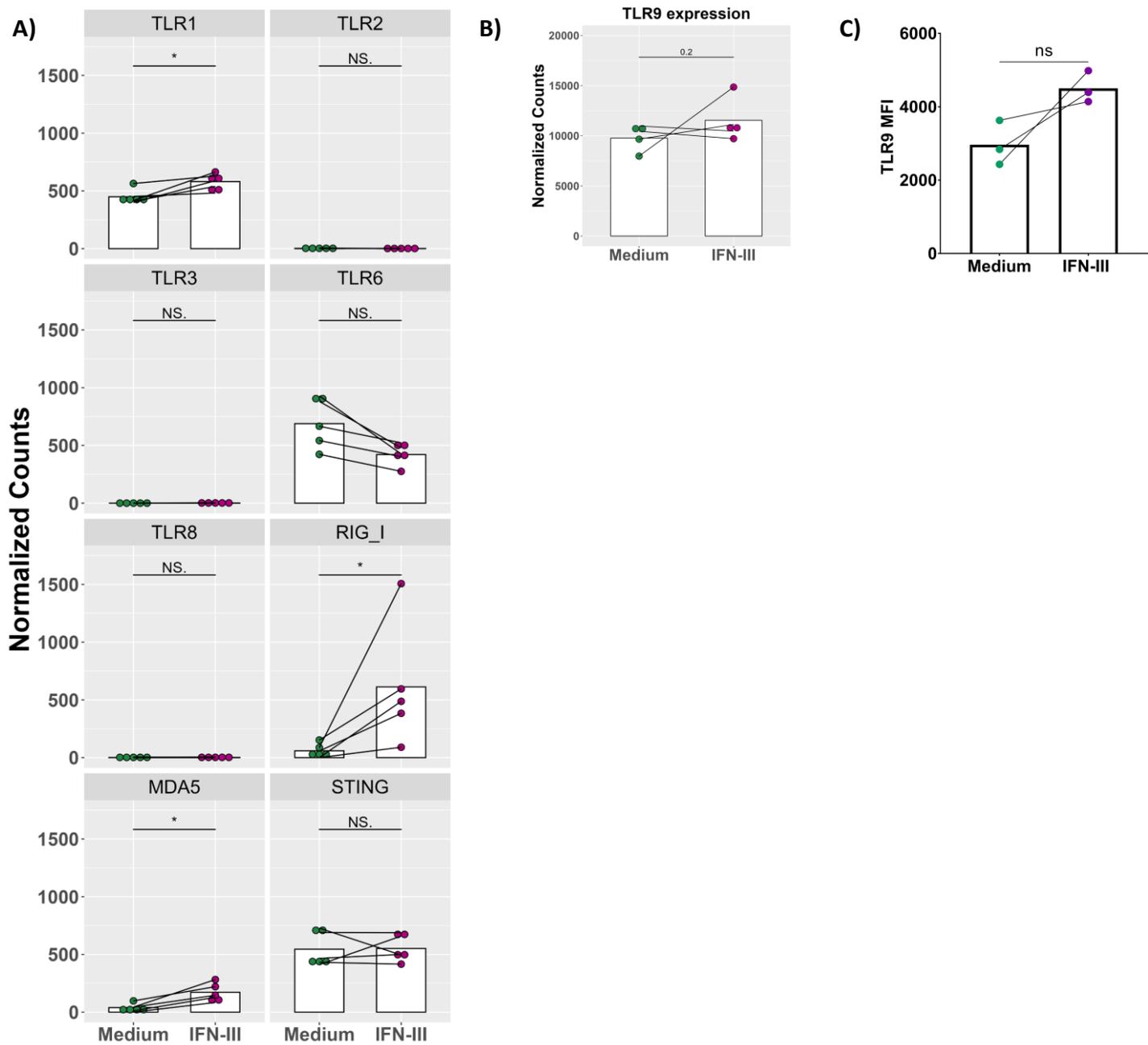
**Supplementary Figure 1. NK cells and T cells do not respond to IFN-III.**

**A&B)** T cells and NK cells pSTAT1 was measured by flow cytometry, after 45min with 100 ng/mL IL-29 or 1,000 U/mL IFN- $\beta$  (n = 5). **C)** pSTAT1 phosphorylation of a representative donor. **D)** pSTAT1 from healthy donors (n=5). Statistical significance was determined with Friedman tests on the MFI values: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



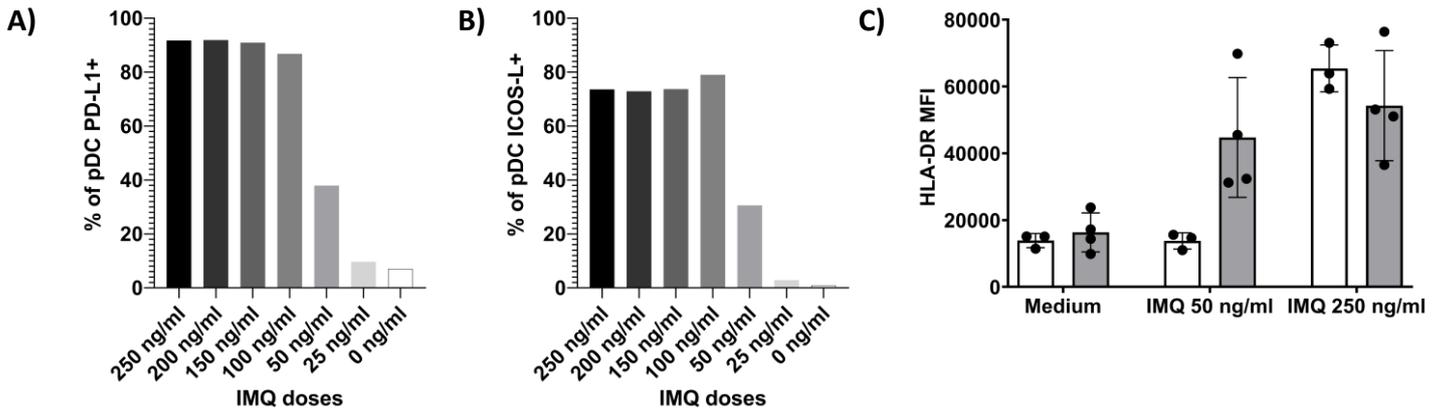
**Supplementary Figure 2. IFN-III induces the strongest activation at 12h and upregulates IFN-γ and IFN-α response pathways.**

**A)** RT-qPCR analysis of OAS2 expression on purified pDCs treated 6h, 12h, or 18h with 100 ng/mL of IL-29. **B)** Venn Diagram plot showing enriched genes present in interferon gamma versus interferon alpha signaling pathways of the msigDB.



**Supplementary Figure 3. IFN-III impact on the expression of TLR, RIG-I, MAD5, and STING.**

**A)** Normalized counts of *TLR1*, *TLR2*, *TLR3*, *TLR6*, *TLR8*, *RIG\_I*, *MDA5*, and *STING* genes from RNA sequencing of purified pDCs treated with or without 100 ng/mL of IL-29 after 12h. Statistical significance was addressed with a Wilcoxon test. **B)** Normalized counts of *TLR9* genes from RNA sequencing on pDCs treated with or without 100 ng/mL of IL-29 after 12h. Statistical significance was addressed with a Wilcoxon test. **C)** TLR9 protein expression was evaluated by flow cytometry purified pDCs treated with or without 100 ng/mL of IL-29 after 18h.



**Supplementary Figure 4. Determination of IMQ suboptimal dose for pDCs activation.**

**A)** PD-L1 and **B)** ICOS-L positive pDCs were quantified by flow cytometry after being cultivated 42 hours with different doses of imiquimod. **E)** HLA-DR MFI was quantified on purified pDCs by flow cytometry after different combination of treatments with or without 100 ng/mL of IL-29 for 18h and 250 ng/mL or 50 ng/mL of IMQ for 24h.



# DISCUSSION



During my PhD fellowship, I explored the role of IFN-III on pDC and its implication in antitumor response. I first defined the positive impact of IFN-III on blood pDCs in terms of activation and functions. Then, I unveiled the role of TLR7 in this activation. The use of IFN-III in combination with TLR7 agonist, would favor the production of innate cytokines in the TME, while reducing the toxic effects of TLR7 agonist injections. Finally, I demonstrated that this IFN-III pathway is sufficient to overcome TGF- $\beta$ -induced inhibition on pDCs that can occur in immunosuppressive TME.

Altogether, our results suggest that targeting TA pDC with a combination of IFN-III and TLR7-L to restore their IFN- $\alpha$  production might be a strategy to induce antitumor immunity. Nevertheless, many questions remain open on the role of pDC and IFN-III in the TME.

## **I. Do IFN-III stimulated pDCs have immunostimulatory or immunomodulatory properties?**

### **A. Role of PD-L1 and ICOS-L expression on pDC**

We demonstrated that IFN-III elicit a strong IFN-I secretion by pDCs after TLR7 activation. Their immunostimulatory properties could be debated because IFN-III also upregulates PD-L1 and ICOS-L, two proteins known to have immunomodulatory effects. Indeed, PD-L1 expression on DCs attenuates T cell activation (Peng et al. 2020), and ICOS-L expression on pDCs contribute to Treg expansion (Faget et al. 2012). Nevertheless, ICOS-L induces Treg expansion in the absence of IFN-I (Faget et al. 2012). Moreover, IFN-I attenuate Treg functions in the TME (Gangaplara et al. 2018). Therefore, even if IFN-III stimulated pDCs express ICOS-L at their surface, their own production of IFN-I may be sufficient to inhibit Treg expansion. Regarding PD-L1, it is also expressed on mature DCs along with DC-LAMP (Maier et al. 2020). Therefore, PD-L1 could define pDC that was activated and reached a terminal phase of maturation. Finally, pDCs exposed to IFN-III may secrete such a high dose of IFN-I that its impact on other immune cells could overcome immunomodulatory effects induced by PD-L1

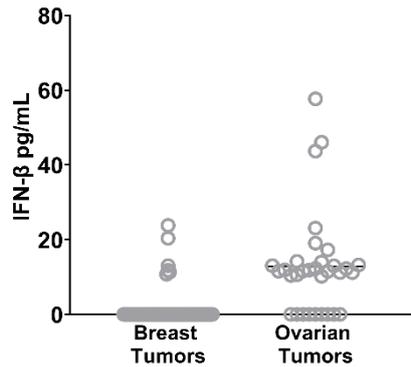
expression. Notably, IFN-I secreted by pDCs is known to enhance cDC antigen presentation capacity (Bencze, Fekete, and Pázmándi 2021).

## **B. pDCs: positive or negative prognosis?**

pDC high infiltration in TME was associated with poor prognosis in breast (Treilleux et al. 2004; Sisirak et al. 2012) and ovarian cancer (Labidi-Galy et al. 2011; 2012), but it was also associated with a good prognosis in colorectal (Kießler et al. 2021), pancreas (Plesca et al. 2022) and breast cancers (Michea et al. 2018; Oshi et al. 2020) **[Table 2]**. Moreover, preliminary data from the team indicate that pDCs infiltration in melanoma tumors is associated with a better response after ICI treatment. This divergence in prognosis can be explained by the analysis of limited tissue microarray slides (TMA) versus whole tumor slides that encompass much wider area and cells. It is therefore still difficult to determine the prognosis of pDC infiltration in tumors.

Mechanistically, a poor prognosis could be linked to ICOS-L expression on pDCs and Treg expansion in absence of IFN-I in the TME (Faget et al. 2012). Unfortunately, pDCs which are the main producer of IFN-I are inhibited TGF- $\beta$  present in the TME (Sisirak et al. 2013). Hence, no IFN-I were quantified in breast and ovarian cancer. Indeed, our laboratory demonstrated that IFN- $\beta$  was absent in 91% of primary dilacerated breast tumors (SN-Dil) (n=67) **[Figure 15]**. In the remaining tumors, the mean concentration of IFN- $\beta$  present in SN-Dil reached only 15 pg/mL. In ovarian tumor, IFN- $\beta$  was absent in 28% of primary dilacerated ovarian tumors (SN-Dil) (n=32), with a mean of IFN- $\beta$  concentration equal to 18 ng/mL in the SN-Dil that do have IFN- $\beta$  **[Figure 15]**.

Altogether these results, suggest that by restoring TA-pDCs secretion of IFN-I through a combination of IFN-III and TLR7 agonists, pDCs will no longer induce Treg expansion. Consequently, it is the pDC state of activation that should be taken in consideration regarding to their prognosis more than their rate of infiltration.



**Figure 15 – Low concentration of IFN-β in breast and ovarian SN-DiL**

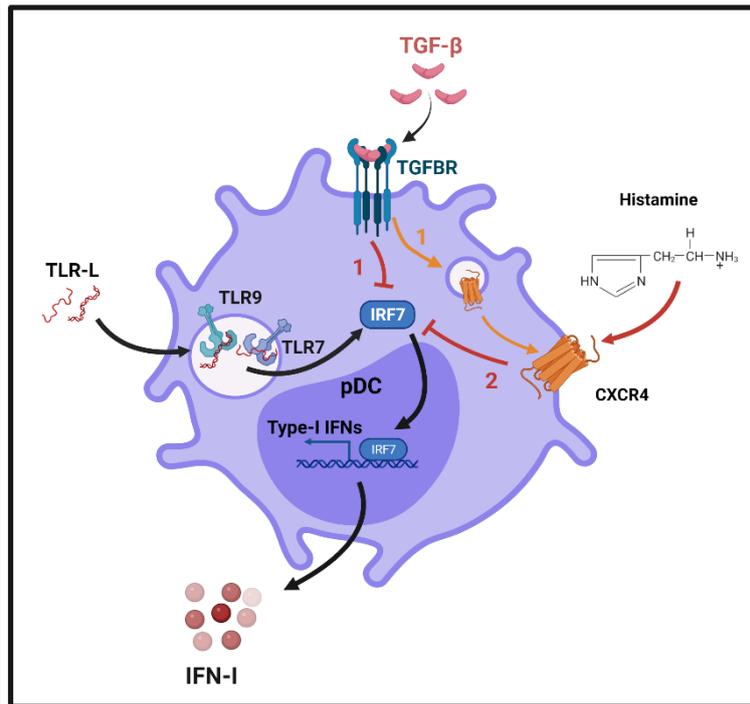
IFN-β concentration was quantified in SN-DiL of 67 breast SN-DiL and 32 ovarian SN-DiL by multiplex assay.

## II. Balance between IFN-III activation and TGF-β inhibition

We used exogenous TGF-β to recreate the TME inhibition of pDCs. To go further, it would be interesting to expose purified pDCs to SN-DiL to correctly mimic TME inhibitory impact on pDCs and test the effect of IFN-III supplementation. Using SN-DiL is important as TA-pDC could also be inhibited by other molecules such as lipids (i.e. PGE2), or enzymes (i.e. arginase1) could lead to an immunosuppressive environment (Arlaukas et al. 2018; Bekeredjian-Ding et al. 2009). Moreover, the mechanism involved in TGF-β counteraction by IFN-III remains unknown. One hypothesis is that IFN-III induces a downregulation of TGFβR1 at the protein level inhibiting TGF-β signaling. IFN-III could also inhibit of TGF-β downstream signaling by an inhibitory intracellular factor. For instance, smad2 and smad3 phosphorylation might be impaired by IFN-III signaling. Another hypothesis would be that IFN-III induction of IFN-I production is so strong in pDCs that TGF-β pathways not sufficient to inhibit IFN-I production.

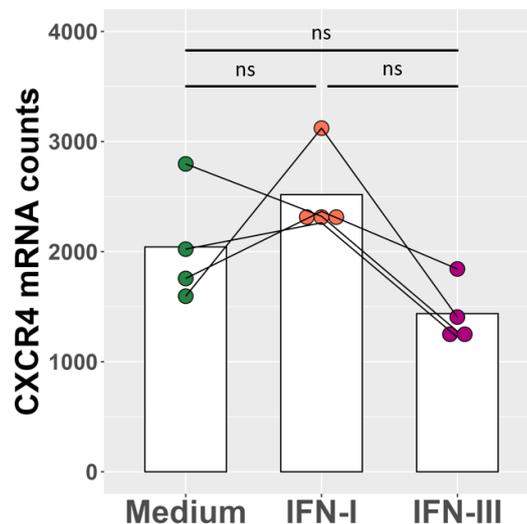
It is also important to discuss the TGF-β – CXCR4 axis. As mentioned in the introduction pDC stimulation with TGF-β increases their expression of CXCR4 (Bekeredjian-Ding et al. 2009). It has been suggested that the purpose of CXCR4 increase was to maintain pDCs in the TME instead of going to the draining lymph node to activate T cells. However, CXCR4 expression could also reinforce TGF-β inhibition on pDCs. Indeed, it has been demonstrated that natural amines such as histamine and clobenpropit, binds CXCR4 (Smith et al. 2017). Actually, pDCs pre-treatment with natural amine inhibit the production of IFN-I that should occur after TLR stimulation (Smith et al. 2017). In terms of mechanistic, the engagement of CXCR4 with the

synthetic compound IT1t, mimicking natural amines, inhibits IRF7 transcription and phosphorylation (Smith et al. 2019). The lack of IRF7 transcription and phosphorylation impair the formation of the ISGF3 complex necessary for ISG transcription and IFN-I production **[Figure 8]**. Undoubtedly, CXCR4 interaction with IT1t inhibits ISG transcription and IFN-I production (Smith et al. 2019). In the TME, natural amines, such as histamine, are highly present. Histamine's impact on tumor-associated immune cells has been reviewed by *Sarasola et al.* where they indicate the antitumoral and pro-tumoral function of histamine according to the immune subset it acts on (Sarasola et al. 2021). Yet the impact of histamine on TA-pDC has to be investigated. However, with these previous studies we could suggest that tumor-derived TGF- $\beta$  increases CXCR4 expression on pDCs and that CXCR4 could be triggered by the histamine present in the TME. This engagement will induce a second inhibitory signal reinforcing TGF- $\beta$  impairment of IFN-I production **[Figure 16]**. Hence, it would be interesting to sequentially treat pDCs with TGF- $\beta$ , then histamine, before TLR7 activation to validate this mechanism of inhibition. Finally, while CXCR4 do not belong to the DEGs recovered from analysis comparing non-stimulated to IFN-III stimulated pDCs, a modulation in its transcription can be observed **[Figure 17]**. IFN-I stimulation seems to slightly upregulate CXCR4 mRNA expression whereas IFN-III decrease its expression. These observations provides an insightful observation concerning the mechanism in which IFN-III counteract TGF- $\beta$  inhibition.



**Figure 16 – TGF- $\beta$  – CXCR4 axis inhibits IFN-I production**

Representation of the hypothesis where (1) TGF- $\beta$  stimulation inhibits IFN-I production through the inhibition of IRF7 and enhance the surface expression of CXCR4 in parallel. CXCR4 available at the pDCs membrane could bind histamine leading to the inhibition of IFN-I production in an IRF7 dependent manner signal (2).



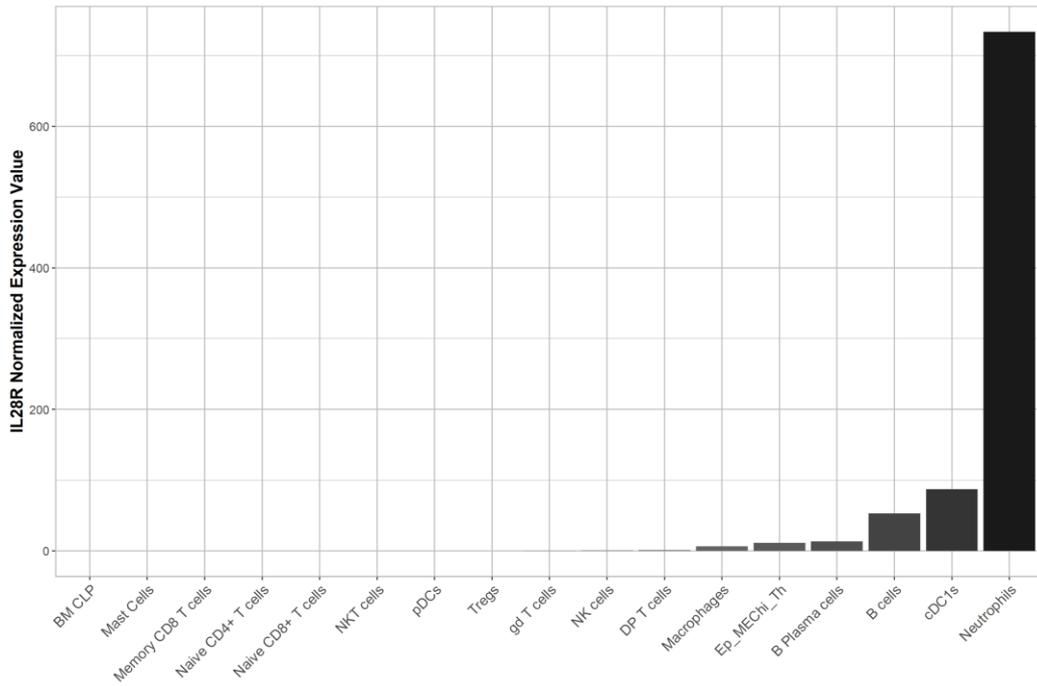
**Figure 17 – Modulation of CXCR4 mRNA expression on IFN-I or IFN-III stimulated pDCs**

CXCR4 normalized counts from RNA sequencing of pDCs treated with IFN-I or IFN-III for 12h. Statistical significance was addressed with a Freidman test.

### III. Do neutrophils respond to IFN-III?

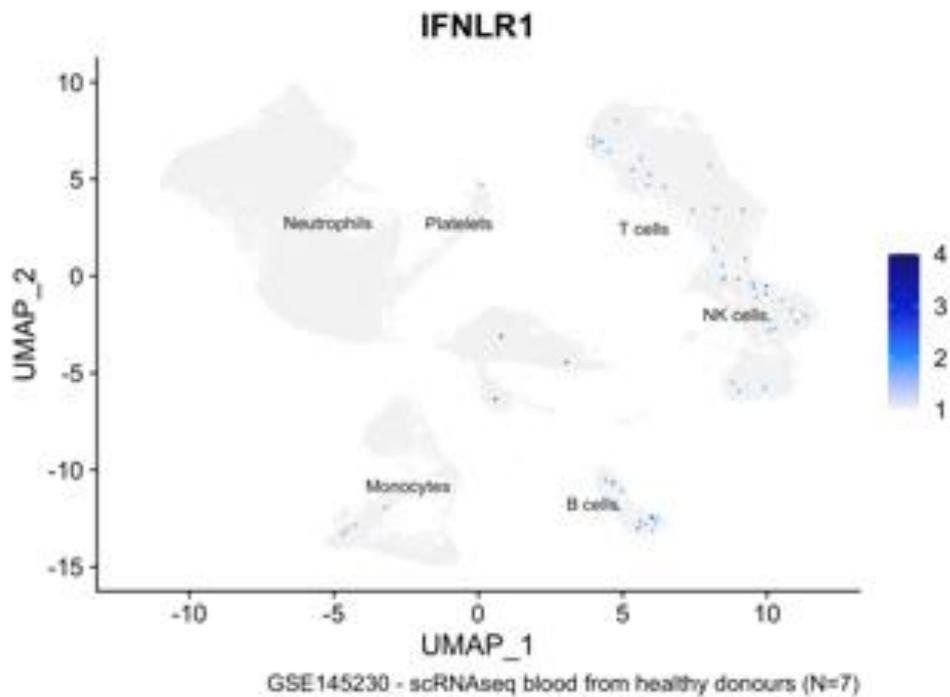
We initially screened *IFN $\lambda$ R1* expression on bulk RNA-seq from human and mice public data. In human, these databases did not include neutrophils. However, mice database included neutrophils [Figure 18]. At steady state in mice, neutrophils are the immune cells that express the highest level of *IFN $\lambda$ R1* gene. cDC1 and B cells also express *IFN $\lambda$ R1* gene, even if their expression levels are 7 times lower compared to neutrophils. Interestingly, pDCs do not express *IFN $\lambda$ R1* gene in mice. This observation can be link to the fact that no studies investigated the role of IFN-III on pDCs in mice. Nevertheless, pDCs response to IFN-III could be induced by viral infection as shown in human (Yin et al. 2012) or maybe by TLR agonists treatment.

To include neutrophils in our screening of *IFN $\lambda$ R1* expression in human. We looked at public data of scRNA-seq that include neutrophils. No *IFN $\lambda$ R1* expression was detected in the neutrophil cluster [Figure 19]. This absence of expression could be explained by the very weak expression of *IFN $\lambda$ R1* gene in immune cells that could not be detected by scRNA-seq with the actual depth of sequencing. Moreover, our results did not include neutrophils but it has been reported that human neutrophils can express *IFN $\lambda$ R1* genes after TLR4 stimulation (Broggi et al. 2017). Hence, at steady state, human neutrophils seem to not express *IFN $\lambda$ R1*.



**Figure 18 – IFN $\lambda$ R1 expression in mice immune cells**

Normalized counts were retrieved from public data available on ImmGen to assess receptor expression of mice immune cells.



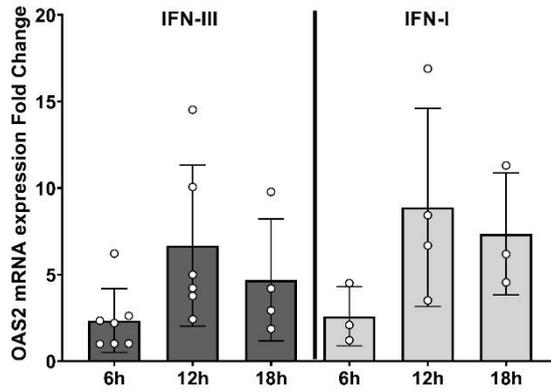
**Figure 19 – IFN $\lambda$ R1 expression in sc-RNA seq of human immune cells**

Normalized counts were retrieved from public data available on ImmGen to assess receptor expression of human immune cells.

## IV. IFN-I induce broader modulations in pDC than IFN-III

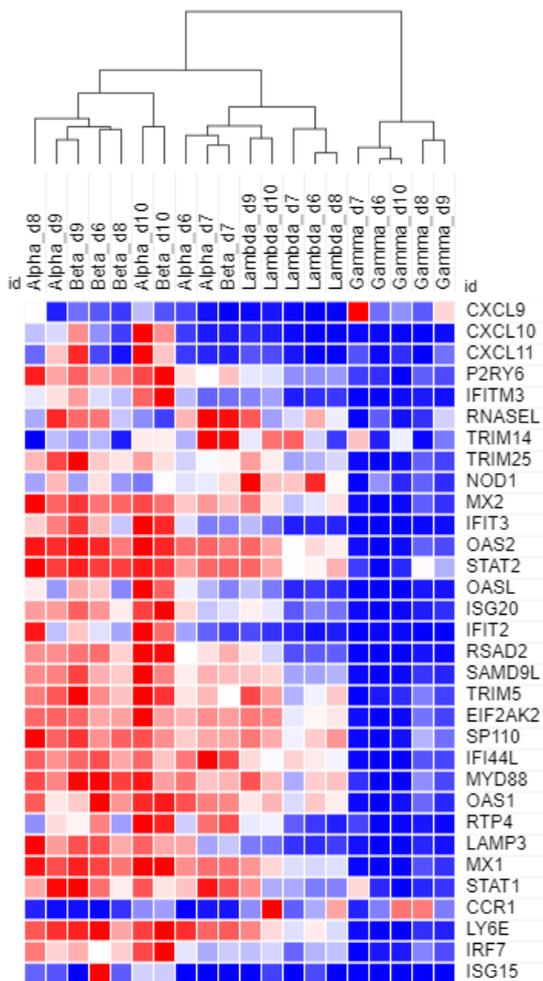
Whereas IFN-I and IFN-III act with different kinetics on epithelial cells [Figure 14], pDCs responded with the same kinetic to IFN-I and IFN-III [Figure 20]. OAS2, a known ISG, reached its higher transcription rate at 12h post-stimulation with IFN-I or IFN-III treatment. However, ISGs induction in pDCs was weaker with IFN-III stimulation compared to IFN-I [Figure 21]. When we compared the enriched pathways after IFN-I or IFN-III stimulation, identical pathways were enriched by both IFN families [Figure 22]. These results led us to believe that IFN-I and IFN-III induced the same functions in pDCs, although to a different extent. However, we identified some pathways that were induced in IFN-I but not in IFN-III. This raised the question whether IFN-I mediate functions that IFN-III do not [Figure 22]. For example, IFN-I induced pathway enrichments of the inflammatory response, apoptosis and cell cycle, whereas IFN-III do not. In mice, IFN-I have been associated with the control of DC turnover, as it controls pDCs apoptosis (Swiecki and Colonna 2015), and HSC differentiation into DC (Essers et al. 2009). Apoptosis and P53 pathways were not enriched when pDCs were stimulated with IFN-III, neither were G2M checkpoint, E2F targets, and MYC targets pathways. This indicated that IFN-I can control pDC turnover contrary to IFN-III. Furthermore, delayed and prolonged secretion of IFN-I were associated with hyperinflammation in severe COVID-19 cases (Y.-M. Kim and Shin 2021). Proinflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-8 were quantified in these severe cases (Galani et al. 2021). This corroborates with our results where IFN-I enriched more pathways involved in inflammation compared to IFN-III. In respiratory viral infection, IFN-III could then induce less inflammation leading to a better outcome (Y.-M. Kim and Shin 2021) suggesting a more fine tune response with IFN-III compared to IFN-I. Finally, in our data, IFN-I enriched pathways also involved glycolysis and hypoxia. The impact of hypoxia and the presence of glucose on myeloid metabolism has been linked to inflammatory response (O'Neill and Pearce 2015).

Altogether, these results highlight similarities but also exclusive features between IFN-I- and IFN-III-induced modulation of pDCs.



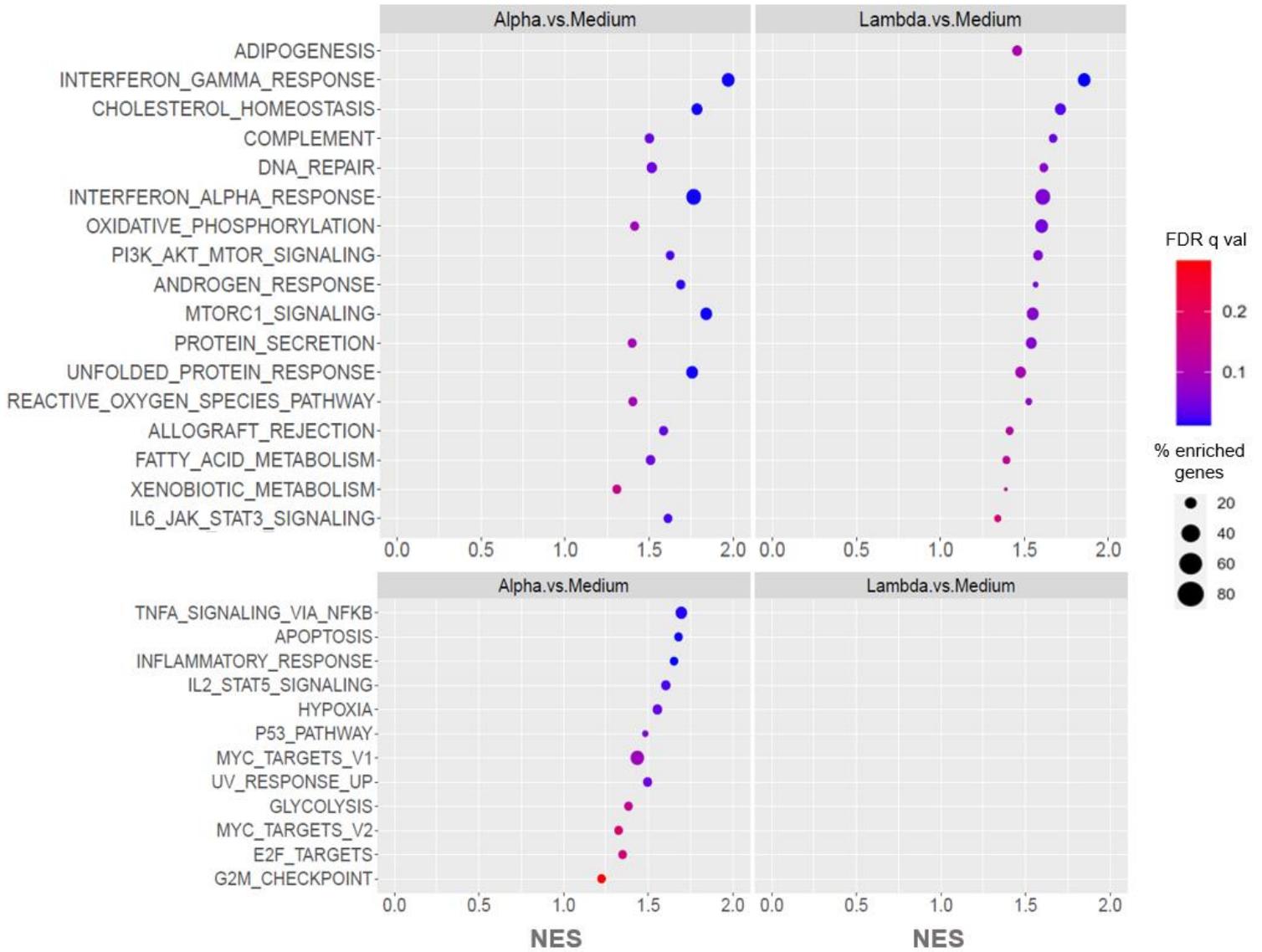
**Figure 20 – IFN-I and IFN-III have the same kinetics of pDCs activation**

RT-qPCR analysis of OAS2 expression on purified pDCs treated 6h, 12h, or 18h with 100 ng/mL of IL-29 (dark grey histograms) or 1000 UI/mL of IFN- $\alpha$ 2 (light grey histograms). Data are depicted as histograms with the mean  $\pm$  S.D and individual values (empty circles). 4 technical replicates were performed per condition.



**Figure 21 – IFN-I induce the strongest ISG induction compared with other IFNs**

Heatmap of z-scores RNA-Seq expression of ISGs. Each column corresponds to a sample of pDCs treated with IFN- $\alpha$ , IFN- $\beta$ , IFN- $\lambda$ , or IFN- $\gamma$ . Each row corresponds to a specific gene. The patient samples are hierarchically clustered (Euclidean distance) over all coding genes.



**Figure 22 – IFN-I but not IFN-III induce pathways involved in cell apoptosis, cell cycle, and inflammation**

GSEA Hallmark analysis showing enriched gene sets. The x-axis represents NES values indicating the hallmark enrichment in IFN-III treated-pDCs or IFN-I treated-pDCs versus non-treated pDCs. FDR q values are represented in a gradient of blue-to-red and the size of each dot represents the percentage of genes enriched for each hallmark.

## **V. Influence of IFN-III and IL-3 on pDCs functions**

### **A. IFN-III and IL-3 influence on their secretome**

On one hand, we demonstrated that pDCs pre-treated with IFN-III gain the capacity to produce many different cytokines and chemokines (IL-6, TNF- $\alpha$ , CX3CL1, MIP1-b). This ability was associated with a specific phenotype: PD-L1+ CD80- pDCs. A synergic action of IFN- $\alpha$  and IL-6 was described to favor B cell differentiation into plasma cells (Jego et al. 2003).

On the other hand, IL-3 increased the proportion of pDCs having a phenotype specific to APC function: PD-L1- CD80+ pDCs. We also demonstrated that when cultivated with IL-3, pDCs with “APC function” have a higher expression of CXCL10, and CCL19. CXCL10 is commonly known to increase CD8+ T cell recruitment in human malignant melanoma lesions (Kunz et al. 1999; Harlin et al. 2009; Dengel et al. 2010). CCL19 is associated with T cell recruitment and T cell activation in HBV (Yan Yan et al. 2021). This supports the concept that IL-3 reinforce pDCs APC properties (Alculumbre et al. 2018; Ito et al. 2001).

### **B. Is pDC maturation accelerated by IL-3?**

pDCs pre-treated with IFN-III produce pro-inflammatory cytokine and IL-3 increased the proportion of pDCs having a phenotype associated to APC functions. This leads to open questions: Do IFN-III and IL-3 induce two different subpopulations of pDCs? Or, is the kinetic of maturation between IFN-III and IL-3 stimulated pDCs different? Indeed, it was suggested that different clusters of pDCs are endowed with different functions at steady state (Ghanem et al. 2022). Besides each stimulus depending on its nature will induce a specific pDCs state (Alculumbre et al. 2018). In particular, GM-CSF induces PD-L1- CD80+ pDC while Influenza virus induces PD-L1+ CD80+ pDC. However, it was demonstrated in mice that pDCs undergo three stages of maturation after viral infection (Abbas et al. 2020). pDCs start by producing IFN-I and continue their maturation until they acquire functional APC properties. Nevertheless, evidence of this pDCs trajectory has yet to be demonstrated in human. In our experiment, we

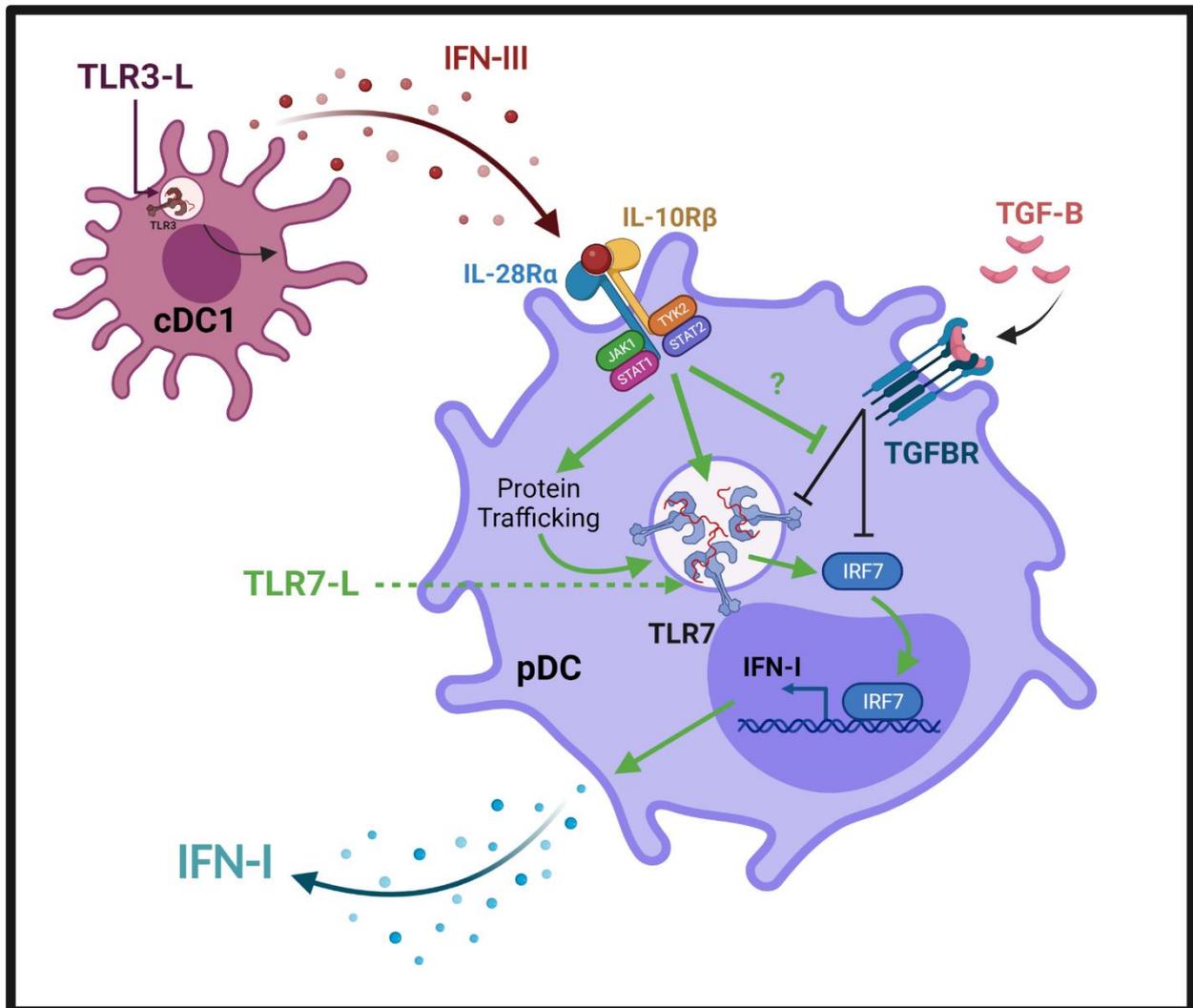
identified three subpopulations of pDCs across different stimulation, but it would have been interesting to analyze these pDCs throughout the course of activation and on a longer period. Indeed, IL-3 stimulated pDCs could have reach their final state of maturation more rapidly than IFN-III stimulated pDCs. We showed that after 42h of culture, a high quantity of IFN-I (8 ng/mL) was present in IL-3 stimulated cells even though only 15% of pDCs had a P1 subpopulation phenotype and 43% a P3 phenotype. This IFN-I production might be due to the small fraction of IFN-I producing pDCs. But it may also be the reminiscence of mature pDCs which went through a stage of IFN-I production during their maturation process.

## **VI. Combining IFN-III with TLR agonist for new immunotherapy strategies**

Our results provide favorable elements concerning the use of IFN-III as an immunotherapy. To further investigate the potential of IFN-III as immunotherapy, we plan to analyze the contribution of IFN-III *in vivo* in the control of tumor growth. The contribution of IFN-III in tumor control could be assessed by monitoring the mammary tumor growth in *IFNAR1*<sup>-/-</sup> deficient mice. It will also be interesting to evaluate the kinetics of IFN-I and IFN-III production during tumor growth in WT mice, but also assess the kinetics of ISG activation on immune and epithelial cells after IFN-I or IFN-III stimulus. Moreover, our results show that IFN-III is most effective in combination with TLR7 agonists. Hence, intratumor injection of TLR7 and IFN-III with tumor growth monitoring could also be performed in WT mice. Finally, to assess if IFN-III antitumor function is mediated by pDCs or DCs in mice, similar experiments should be done in mice lacking each of the DC populations (karma mice (Alexandre et al. 2016); BDCA2-DTR mice). Such experiments would increase our understanding of the precise role of IFN-III in antitumor immunity.

The long-term goal would be to combine IFN-III and TLR7 agonist together. This strategy would allow to use lower doses of TLR7 agonists and then potentially limit classical associated severe adverse events.

## I. General conclusion



**Figure 23 – IFN-III prime pDCs to lower their threshold of activation by TLR7 ligand and thus increase their production of IFN-I**

cDC1 are the only immune cells within the TME to produce IFN-III upon TLR3 stimulation. After stimulation with IFN-III, pDCs upregulate their expression of TLR7 protein and of genes associated to pathways involved in TLR7 trafficking and activation. This priming enhances pDC response to TLR7 ligands (TLR7-L), and induces IFN-I secretion.



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