

Endogenously produced TNF- α contributes to the expression of CXCL10/IP-10 in IFN- λ 3-activated plasmacytoid dendritic cells

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ABSTRACT

The interplay between IFN- λ s and dendritic cells is becoming increasingly relevant, particularly in light of their key role in inducing the antiviral state, including in hepatitis C virus infection. In this work, we have analyzed extensively how human plasmacytoid dendritic cells respond to IFN- λ 3. We report that plasmacytoid dendritic cells incubated with IFN- λ 3 prolong their survival; alter their expression pattern of surface HLA-DR α , CD123, CD86, and CD303; and time dependently produce IFN- α , CXCL10/IFN- γ -induced protein 10, and even modest quantities of TNF- α . Nevertheless, endogenously produced TNF- α , but not IFN- α , was found to be essential for driving the expression of CXCL10/IFN- γ -induced protein 10 in IFN- λ 3-treated plasmacytoid dendritic cells, as revealed by neutralizing experiments by use of adalimumab, etanercept, and infliximab. We also observed that based on the kinetics and levels of IFN- α and CXCL10/IFN- γ -induced protein 10 produced by their IFN- λ 3-treated plasmacytoid dendritic cells, healthy donors could be categorized into 2 and 3 groups, respectively. In particular, we identified a group of donors whose plasmacytoid dendritic cells produced modest quantities of CXCL10/IFN- γ -induced protein 10; another one whose plasmacytoid dendritic cells produced elevated CXCL10/IFN- γ -induced protein 10 levels, already after 18 h, declining thereafter; and a 3rd group characterized by plasmacytoid dendritic cells releasing very high CXCL10/IFN- γ -induced protein 10 levels after 42 h only. Finally, we report that in plasmacytoid dendritic cells, equivalent concentrations of IFN- λ 3 and IFN- λ 1 promote survival, antigen modulation, and cytokine production in a comparable manner and without acting additively/synergistically. Altogether, data not only

extend the knowledge on the biologic effects that IFN- λ s exert on plasmacytoid dendritic cells but also add novel light to the networking between IFN- λ s and plasmacytoid dendritic cells in fighting viral diseases. *J. Leukoc. Biol.* 99: 107–119; 2016.

Introduction

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

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

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

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

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their corresponding receptor subunits, it is clear that type I and type III IFN antiviral actions do not merely duplicate but probably complement each other [2, 3, 12]. All IFN- λ s signal through the same heterodimeric receptor complex composed of a unique IFN- λ R1 (also known as IL-28R α or cytokine receptor family 2 member 12) chain and IL-10R2 [3, 13]. Whereas IL-10R2 is ubiquitously expressed, IFN- λ R1 displays a restricted tissue expression that is limited to epithelial cells of the respiratory, gastrointestinal, and reproductive tracts or to hepatocytes [14, 15]. Interestingly, in cells of the immune system, only pDCs and less prominently, B cells, express IFN- λ R1 [16–18], but only pDCs have been unequivocally shown to respond to IFN- λ s, in terms of altered CD80 expression [16], STAT1 phosphorylation activation [17], and MX1 mRNA induction [19].

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

In this study, we have analyzed extensively how human pDCs respond upon incubation with IFN- λ 3 and show that IFN- λ 3-treated pDCs survive longer, undergo a partial maturation, and produce IFN- α , CXCL10/IP-10, and TNF- α . We also show that even though CXCL10/IP-10 totally depends on endogenously secreted TNF- α , donor-dependent factors likely condition, in a differential manner, the production of IFN- α and CXCL10/IP-10 by IFN- λ 3-treated pDCs.

MATERIALS AND METHODS

Cell isolation and culture

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

Cell stimulation

pDCs (0.5×10^5) in 100 μ l were usually plated in 96-well U-bottom plates (Costar; Corning, Corning, NY, USA), incubated in the presence or absence of

usually 30 ng/ml IFN- λ 3 (R&D Systems, Minneapolis, MN, USA), 30 ng/ml IFN- λ 1 (R&D Systems), 20 ng/ml IL-3 (Miltenyi Biotec), 5 μ M R837 (InvivoGen, San Diego, CA, USA), 100 U/ml IFN- α (Pegasys; Genentech, South San Francisco, CA, USA), and 0.1–10 ng/ml TNF- α (PeproTech, Rocky Hill, NJ, USA) and then cultured at 37°C, 5% CO₂ atmosphere, for the times indicated. In selected experiments, pDCs were preincubated for 15 min with 5 μ g/ml ETA (a dimeric fusion protein that consists of the extracellular ligand-binding portion of the human 75 kDa TNFR linked to the Fc portion of the human IgG₁, ENBREL; Amgen, Thousand Oaks, CA, USA), 2.5 μ g/ml ADA (a human-derived rIgG₁ mAb, HUMIRA; Abbott Laboratories, Abbott Park, IL, USA), 2 μ g/ml infliximab (a mouse/human chimeric IgG₁ mAb, REMICADE; Janssen Biotech, Horsham, PA, USA), or their isotype control antibodies (human IgG₁; eBioscience, San Diego, CA, USA), as well as 5 μ g/ml α IFNAR (PBL InterferonSource, Piscataway, NJ, USA) or their isotype control antibodies (mouse IgG_{2a}; R&D Systems), before treatment. In other experiments, 0.125×10^6 CD14⁺ monocytes in 50 μ l were plated in 96-well flat-bottom plates in the presence or absence of pDC-derived supernatants or 0.1 ng/ml TNF- α . After 1 h, cells were collected and centrifuged at 400 *g* for 5 min. Supernatants were harvested and frozen immediately at –80°C, while the corresponding cell pellets were used for flow cytometry analysis or lysed for RNA extraction.

Flow cytometry analysis

To perform phenotypic studies [32], pDCs were first treated with 5% human serum and then stained for 20 min at room temperature by use of the following mAbs: FITC anti-CD303 (Miltenyi Biotec), PE-Cy7 anti-CD123 (BioLegend, San Diego, CA, USA), APC anti-human CD62L (Miltenyi Biotec), APC-Cy7 anti-HLA-DR α (BioLegend), PE anti-CD86 (BioLegend), anti-CD83 (Miltenyi Biotec), and their related isotype controls. For IFN- λ R1 detection, we used 2 μ g/ml PE anti-IFN- λ R1 and as isotype control antibody, PE mouse IgG_{2a} (both from BioLegend), whereas IL-10R2 expression was assessed by indirect staining by use of 10 μ g/ml of an unconjugated mouse anti-IL-10R2 mAb [clone 4B2; kindly provided by Dr. K. W. Moore (when affiliated with DNAX Institute, Palo Alto, CA, USA)] or its isotype control mAb (unconjugated mouse IgG₁; BioLegend), followed by an incubation with 4 μ g/ml secondary PE goat anti-mouse polyclonal antibodies (BioLegend) [33]. Sample fluorescence was then measured by use of an 8-color MACSQuant analyzer (Miltenyi Biotec) and data analysis performed by FlowJo software, version 8.8.6 (Tree Star, Ashland, OR, USA). Phenotypic cell analysis under the various experimental conditions was performed in live cells, identified as singlet Vybrant DyeCycle Violet-negative cells (Life Technologies, Carlsbad, CA, USA) the overall gating strategy for live cells is depicted in Supplemental Fig. 1A [32].

Gene-expression studies

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

Cytokine measurement

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

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

Immunoblots

pDCs (100,000) were incubated with or without 30 ng/ml IFN- λ 3 for 45 and 90 min before blocking the stimulation in ice-cold PBS, supplemented with 2 mM diisopropylfluorophosphate and phosphatase inhibitors (10 mM NaF, 1 mM Na₃VO₄, 10 mM Na₄P₂O₇). Whole-cell extracts were prepared and subjected to immunoblots by standard procedures [31] by use of 1:1000 rabbit polyclonal antibody anti-phospho-STAT2 (Tyr689; Millipore, Darmstadt, Germany), 1:1000 anti-phospho-STAT1 (Tyr701) rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), and 1:500 anti-total-STAT1 or anti-total-STAT2 rabbit polyclonal antibodies (both from Santa Cruz Biotechnology, Dallas, TX, USA). Blotted proteins were detected and quantified by use of the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

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

RESULTS

IFN- λ 3 promotes survival and antigen modulation in human pDCs

Initial experiments confirmed that pDCs, freshly isolated from the peripheral blood of healthy donors, display both subunits composing the IFN- λ R, namely IFN- λ R1 and IL-10R2 (Supplemental Fig. 1B) [17, 18, 34], as well as tyrosine phosphorylated STAT1 and STAT2 if incubated with IFN- λ 3 (Supplemental Fig. 1C). In these latter, as well as in all subsequent, experiments, IFN- λ 3 was used at 30 ng/ml. This was based on preliminary dose-response studies on gene-expression induction (Supplemental Fig. 2A), surface antigen modulation (Supplemental Fig. 2B), and survival (Supplemental Fig. 2C), which identified such concentration as the optimal one to evaluate the effects of IFN- λ 3 in pDCs, in line with other studies [16–19].

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

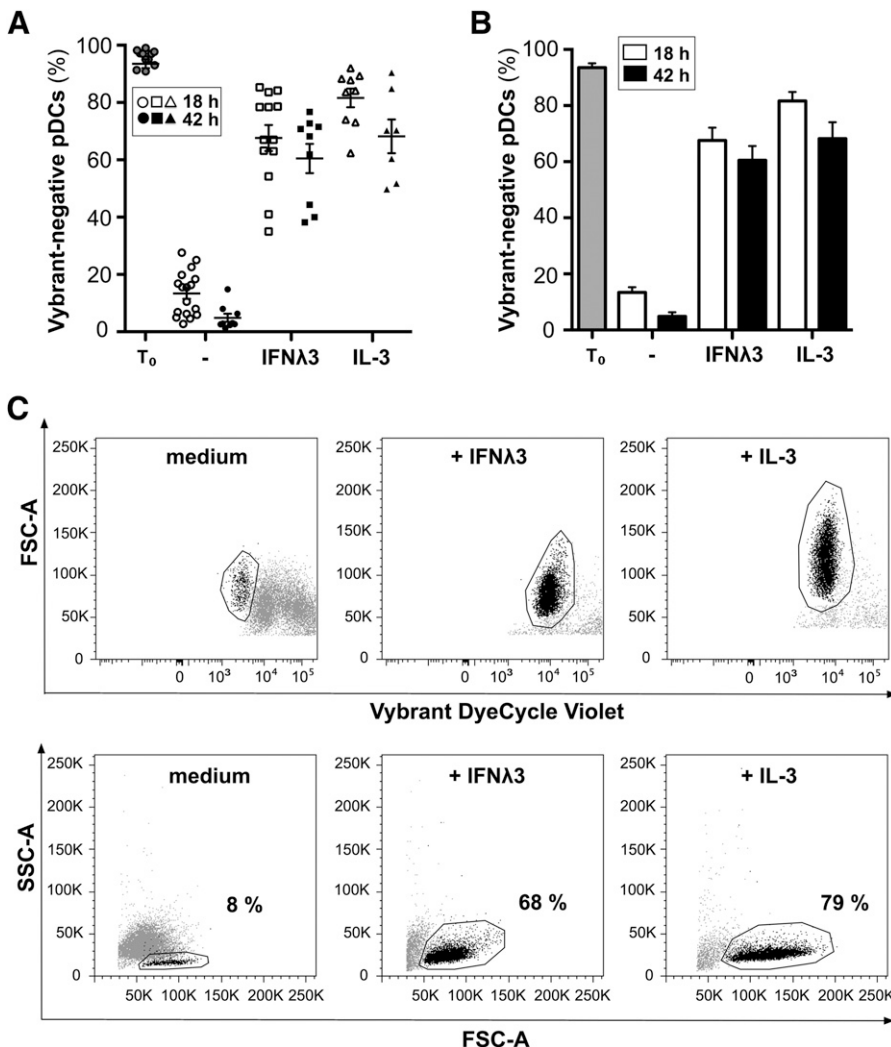


Figure 1. IFN- λ 3 prolongs the survival of pDCs. pDCs were incubated with or without 30 ng/ml IFN- λ 3 or 20 ng/ml IL-3 right after isolation from the blood. Cells were harvested at the 18 and 42 h time points and then stained by the Vybrant DyeCycle to assess their viability compared with freshly isolated pDCs (T₀) by flow cytometry analysis. (A) Results of all individual experiments in terms of viable cells for the conditions displayed ($n = 8$ –16); (B) related means \pm SEM. (C) Gating strategy used to identify pDCs incubated for 42 h that are live, from a representative experiment. (Upper) Viable pDCs gated as Vybrant DyeCycle-negative cells; (lower) cellular morphology and percentages. Gray and black dots stand for dead and live cells, respectively. The overall gating strategy is depicted in Supplemental Fig. 1. FSC-A, Forward-scatter-area; SSC-A, side-scatter-area.

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

42 h, respectively. On the other hand, IL-3 was significantly less efficient than IFN- λ 3 in up-regulating CD83 after 18 h (Fig. 2C).

Taken together, data demonstrate that IFN- λ 3 potently acts on pDCs in terms of enhanced survival and modulation of surface markers. Data also indicate that at least phenotypically [35, 38], IFN- λ 3 induces a partial maturation of pDCs.

IFN- λ 3 induces the production of IFN- α and CXCL10/IP-10 by human pDCs

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

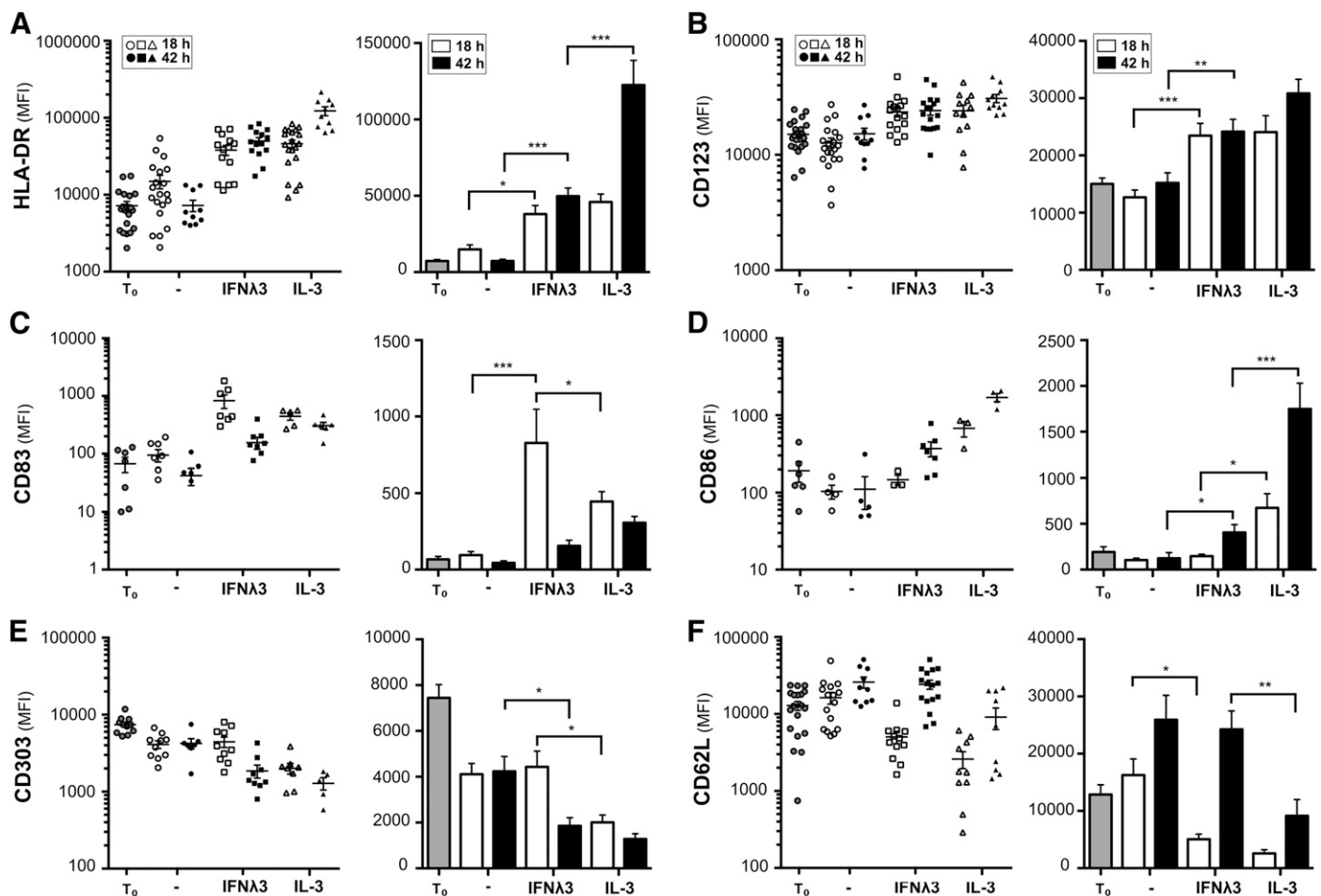


Figure 2. IFN- λ 3 modulates the expression of several antigens in pDCs. After isolation, pDCs were incubated with or without 30 ng/ml IFN- λ 3 or 20 ng/ml IL-3. At the 18 and 42 h time points, cells were harvested and analyzed for HLA-DR (A), CD123 (B), CD83 (C), CD86 (D), CD303 (E), and CD62L (F) expression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

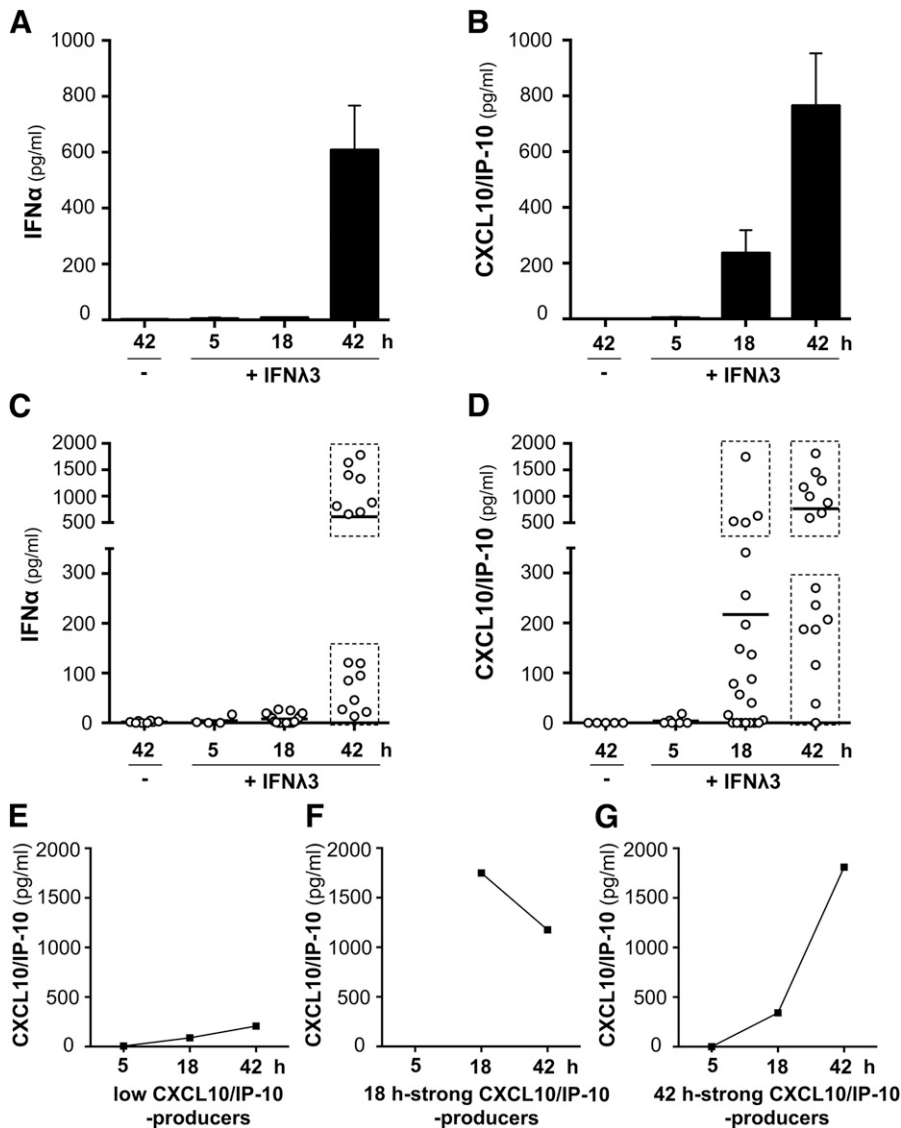


Figure 3. IFN- λ 3 induces a time-dependent production of IFN- α and CXCL10/IP-10 by pDCs.

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

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

Although the graphical representations displayed in Fig. 3A and B (reporting the means \pm SEM of IFN- α and CXCL10/IP-10 release calculated from all samples) would suggest that maximal production of IFN- α and CXCL10/IP-10 by pDCs would occur after 42 h incubation with IFN- λ 3, that was not always the case for CXCL10/IP-10. In fact, we observed a very large variability in the levels of extracellular IFN- α (Fig. 3C) and CXCL10/IP-10 (Fig. 3D) measured in supernatants harvested from pDCs treated with IFN- λ 3 (ranging from a few up to thousands picograms/milliliter), which, at least in some samples for CXCL10/IP-10, already reached their maximum at 18 h (Fig. 3D). More interestingly, we could retrospectively identify 3 reproducible patterns of

CXCL10/IP-10 production by pDCs incubated with IFN- λ 3 (Fig. 3D; dashed boxes), as better illustrated in representative experiments shown in Fig. 3E–G and globally summarized in Fig. 4A–C: a first group, herein referred to donors defined as “low CXCL10/IP-10 producers,” characterized by a modest production of CXCL10/IP-10 at 18 h (22 ± 11 pg/ml, $n = 10$), which even if remaining substantially low, significantly increases at 42 h (163 ± 24 pg/ml, $n = 7$; Figs. 3E and 4A); a second group, herein referred to donors defined as “18 h strong CXCL10/IP-10 producers,” characterized by remarkably elevated levels of CXCL10/IP-10 production already after 18 h (865 ± 297 pg/ml, $n = 4$), which do not further increase at 42 h (722 pg/ml, $n = 2$; Figs. 3F and 4B); and finally, a 3rd group, herein referred to donors defined as “42 h strong CXCL10/IP-10 producers,” characterized by very high CXCL10/IP-10 levels detectable after 42 h (1320 ± 264 pg/ml, $n = 7$; Figs. 3G and 4C).

Similarly to CXCL10/IP-10, 2 patterns of IFN- α production by IFN- λ 3-treated cells were also reproducibly distinguishable based on the extracellular cytokine levels measured at the 42 h time

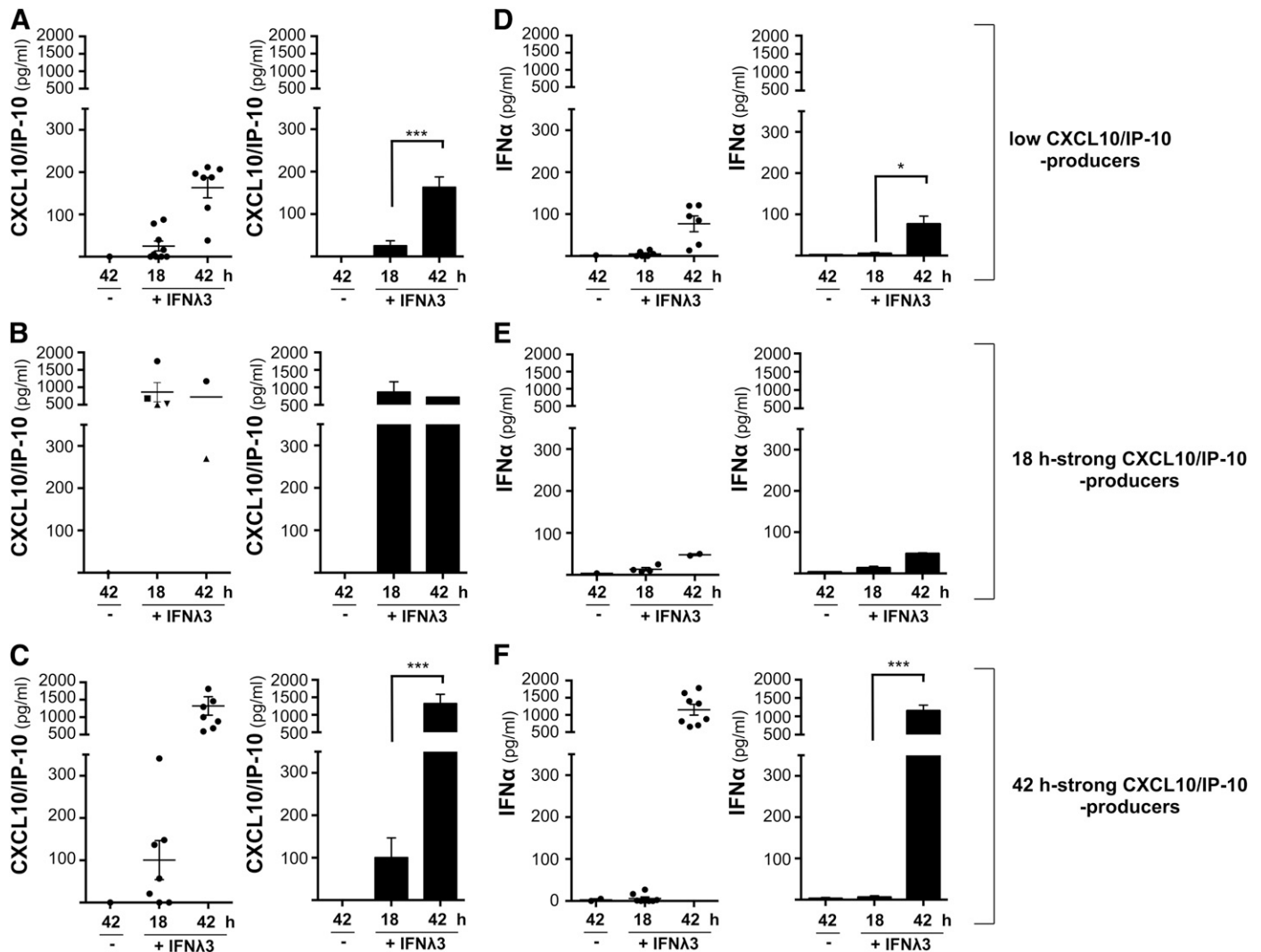


Figure 4. Relationship between the levels of CXCL10/IP-10 and the levels of IFN- α induced by IFN- λ 3 in pDCs. (A and D) Extracellular production of CXCL10/IP-10 and IFN- α , respectively, measured in pDC-derived supernatants harvested from all low CXCL10/IP-10 producers. (B and E) Extracellular production of CXCL10/IP-10 and IFN- α , respectively, from the 18 h strong CXCL10/IP-10 producers. (C and F) Extracellular production of CXCL10/IP-10 and IFN- α , respectively, from the 42 h strong CXCL10/IP-10 producers. For each group, left panels report the absolute values of CXCL10/IP-10 (A–C) and IFN- α (D–F) production by IFN- λ 3-treated pDCs (from all individual experiments); while the right panels (bar graphs) display the means \pm SEM of the values reported in the left ones. pDCs incubated in the absence of IFN- λ 3 for up to 42 h produced neither IFN- α nor CXCL10/IP-10. Please note that in B, each single experiment is identified by the same symbol. Significant increases: * $P < 0.05$, *** $P < 0.001$.

point (Fig. 3C): 1 of them displaying IFN- α amounts <150 pg/ml (Fig. 3C, upper dashed box; here defined as “low IFN- α producers”) and the other 1 >500 pg/ml (Fig. 3C, lower dashed box; here defined as “strong IFN- α producers”). Interestingly, by matching the amounts of IFN- α and CXCL10/IP-10, measured in the same samples (Fig. 4), it seemed evident that the donors whose pDCs produced low levels of IFN- α (Fig. 4D) mainly corresponded to the low CXCL10/IP-10 producers (Fig. 4A), a few of them (Fig. 4E) coinciding with the 18 h strong CXCL10/IP-10 producers (Fig. 4B), whereas the donors whose pDCs produced very high levels of IFN- α (Fig. 4F) all corresponded to the 42 h strong CXCL10/IP-10 producers (Fig. 4A). Such a correspondence was corroborated by calculating the Pearson correlation coefficient, which proved that the release of

CXCL10/IP-10 and IFN- α by IFN- λ 3-treated pDCs was statistically correlated after 42 ($r = 0.683$, $P < 0.01$) but not after 18 ($r = 0.219$, $P = 0.313$) h of incubation. Furthermore, the percentage of live (Vybrant-negative) pDCs after 18 or 42 h of incubation with IFN- λ 3 was found to be substantially similar within the 3 CXCL10/IP-10 (Fig. 5A) or the 2 IFN- α - (Fig. 5B) producer groups, indicating that the variable CXCL10/IP-10 and IFN- α production was not related to differences in pDC viability/death.

Taken together, data not only prove that pDCs treated with IFN- λ 3 produce and release significant quantities of IFN- α and CXCL10/IP-10, but also uncover that the extent of IFN- α and CXCL10/IP-10 production by IFN- λ 3-treated pDCs is very variable and likely influenced by donor-dependent factors.

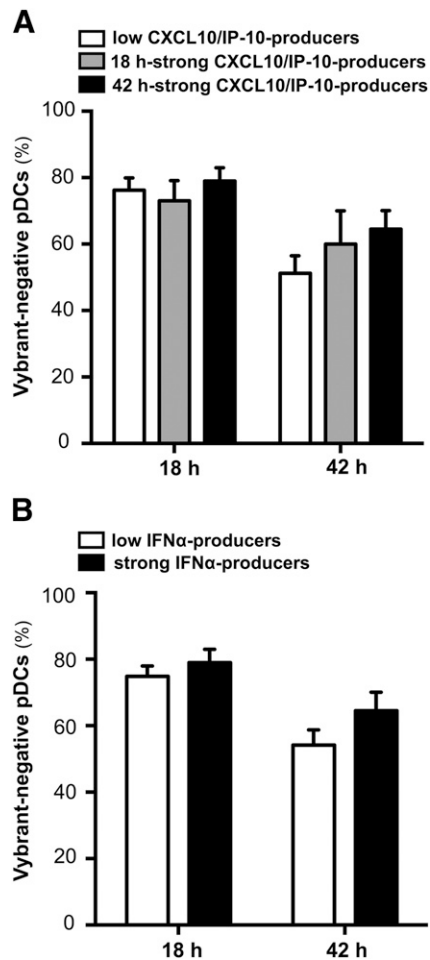


Figure 5. Survival of pDCs treated with IFN- λ 3 categorized according to the CXCL10/IP-10 or IFN- α producer groups. Viability of pDCs after 18 and 42 h of incubation with 30 ng/ml IFN- λ 3, as analyzed by flow cytometry. The percentage of live (Vybrant-negative) pDCs within the 3 CXCL10/IP-10 producer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

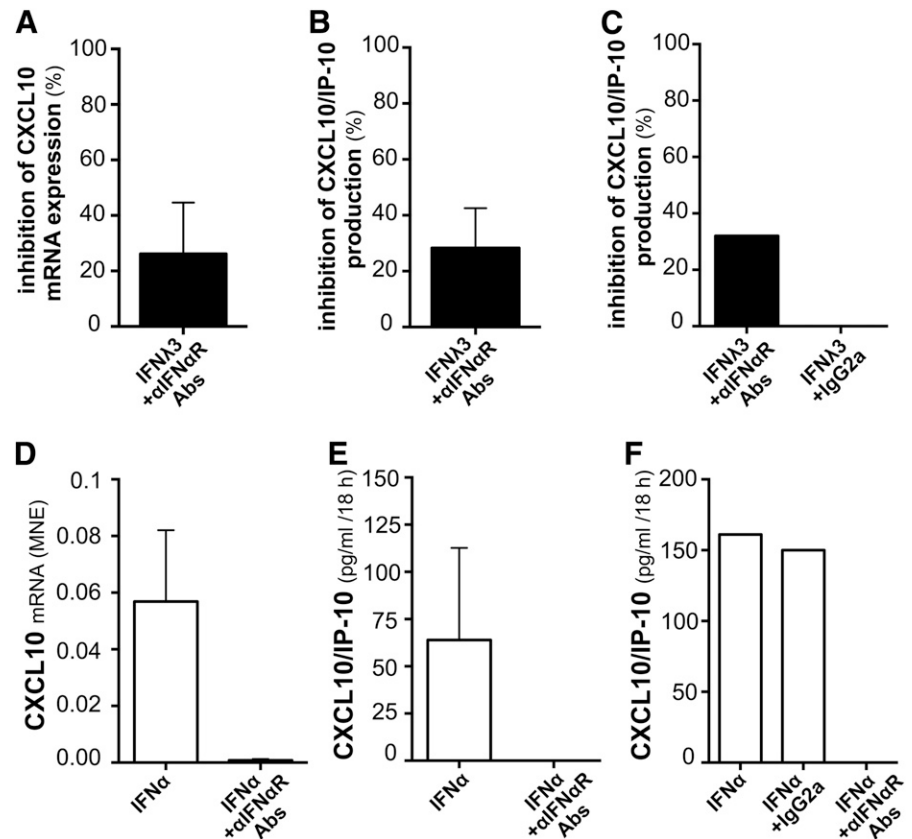
Endogenous IFN- α is only partially required to induce CXCL10/IP-10 in IFN- λ 3-treated pDCs

To ascertain definitively whether the induction of CXCL10/IP-10 expression in IFN- λ 3-treated pDCs depends on endogenous IFN- α , we performed experiments in which pDCs were pre-treated with antibodies neutralizing the IFN- α R [42] before their incubation with IFN- λ 3 (Fig. 6). Luckily, the donors used in these experiments happened to fall, by chance, into the 42 h strong CXCL10/IP-10 producer group. In these experiments, α IFN- α R antibodies only modestly inhibited the induction of CXCL10 mRNA at 18 h (Fig. 6A) or the production of CXCL10/IP-10 at 42 h (Fig. 6B), whereas they completely blocked the induction of CXCL10/IP-10 production and mRNA expression by pDCs incubated with IFN- α (Fig. 6D and E). Under the same experimental conditions, isotype control antibodies did not affect CXCL10/IP-10 production induced by IFN- λ 3 and IFN- α (Fig. 6C and F). Taken together, data demonstrate that CXCL10/IP-10 produced by pDCs after 42 h of incubation with IFN- λ 3 is only partially controlled by endogenous IFN- α .

Endogenous TNF- α is crucial for the induction of CXCL10/IP-10 in IFN- λ 3-treated pDCs

It has been demonstrated that under several conditions [43–45], transcription of CXCL10 can be cooperatively induced by STAT1-activating stimuli (such as type I and type II IFNs), acting in combination with NF- κ B-activating agonists, including TNF- α . Human pDCs are known to produce TNF- α , for instance in response to R837 (Imiquimod) [46, 47]. Therefore, we investigated whether IFN- λ 3 could trigger the production of TNF- α in pDCs and if so, whether TNF- α could endogenously play a role in activating CXCL10/IP-10 expression. As shown in Fig. 7A and B, very low but detectable amounts of TNF- α could be measured in supernatants harvested from untreated and IFN- λ 3-treated pDCs, in the latter case, slowly increasing after 18 h and reaching significant levels up to 42 h. Concomitantly, TNF- α mRNA accumulation significantly increased at 18 h in IFN- λ 3-treated pDCs (Fig. 7C). Notably, the yields of TNF- α were not as variable as the yields of IFN- α and CXCL10/IP-10 measured in the same supernatants, as also confirmed by the coefficient of variability analysis (data not shown) [48]. Despite of these low TNF- α amounts, pDC incubation in the presence of TNF- α blockers, including ETA, ADA, or infliximab [49], completely prevented the induction of CXCL10 mRNA at 18 h (Fig. 7D, and data not shown), as well as of CXCL10/IP-10 production at 18 and 42 h (Fig. 7E, and data not shown), in response to IFN- λ 3, regardless of the "CXCL10/IP-10 producer" group. Under the same experimental conditions, isotype control antibodies did not affect, in pDCs treated with IFN- λ 3, either the production of CXCL10/IP-10 or the modulation of membrane markers (data not shown). On the other hand, ETA inhibited neither the survival of pDCs incubated with IFN- λ 3 for 42 h (Supplemental Fig. 4A) nor the production of CXCL10/IP-10 induced by R837 in pDCs (Supplemental Fig. 4B), whereas both ETA and ADA completely suppressed the induction of CXCL8 mRNA triggered by 10 ng/ml TNF- α in pDCs (Supplemental Fig. 4C). Surprisingly, TNF- α alone, at doses ranging from 0.01 to 10 ng/ml, was found unable or able to trigger only minute amounts of CXCL10/IP-10 by pDCs cultured for up to 42 h (data not shown). However, supernatants harvested from pDCs, incubated for 18 h with IFN- λ 3, but not with medium only, once transferred to CD14 $^{+}$ monocytes for 1 h in the presence or absence of TNF- α inhibitors, induced a TNF- α -dependent CCL4 (Fig. 8A) and I κ B α (Fig. 8B) mRNA expression. Importantly, neither was the amounts of TNF- α contained in these supernatants (\sim 15 pg/ml) further increased once added to CD14 $^{+}$ monocytes nor was TNF- α mRNA induced by them (data not shown), proving that the biologic effects mediated by pDC-derived supernatants were promoted by the TNF- α exclusively derived from IFN- λ 3-treated pDCs. Moreover, whereas addition of 0.1 ng/ml TNF- α to CD14 $^{+}$ monocytes induced CCL4 (Fig. 8C) and I κ B α (Fig. 8D) mRNA expression, addition of IFN- λ 3 neither did so nor potentiated the action of TNF- α (Fig. 8C and D), confirming that monocytes do not respond to IFN- λ 3. Taken together, data demonstrate that IFN- λ 3 induces the production of biologically active TNF- α by pDCs. Data also prove that endogenously produced TNF- α is essential for the induction of CXCL10/IP-10 in IFN- λ 3-treated pDCs.

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



IFN- λ 1 and IFN- λ 3 are equally effective in promoting survival, antigen modulation, and cytokine production in pDCs

In a final series of experiments, we also addressed the potency of IFN- λ 3, relative to that of IFN- λ 1, on pDC survival and/or antigen modulation, as IFN- λ 1 has been shown to counteract the proapoptotic effect exerted in pDCs by DEX [17], as well as to enhance their CCR7, CD62L, CD80, CD83, ICOS-L, and MHC-I expression levels [16, 17]. pDCs were cultured with or without 30 or 100 ng/ml IFN- λ 1 in the presence or absence of 30 ng/ml IFN- λ 3 (purchased from the same company). As shown in **Fig. 9**, the modulatory effects by 30 ng/ml IFN- λ 3 or 30 ng/ml IFN- λ 1 on pDC viability (**Fig. 9A**), as well as on pDC expression of CD86, CD83, and HLA-DR α (**Fig. 9B**), were found to be substantially similar. Furthermore, a combination of the 2 IFN- λ types did not provoke any additive/synergistic effects either (**Fig. 9A and B**). Maximal effects on both cell viability (**Fig. 9A**) and antigen levels (**Fig. 9B**) did not significantly change if IFN- λ 1 were used at 100 ng/ml, consistent with the data obtained with IFN- λ 3 (**Supplemental Fig. 2**).

We also measured the levels of CXCL10/IP-10, IFN- α , and TNF- α in supernatants from the pDCs used for these experiments, which retrospectively involved 3 donors belonging to the 42 h strong CXCL10/IP-10 producers. As shown in **Fig. 9C**, production of CXCL10/IP-10, IFN- α , and TNF- α by pDCs incubated for 42 h with 30 ng/ml IFN- λ 1 was not significantly different from that triggered by 30 ng/ml IFN- λ 3. Once again, a combination of IFN- λ 1 and IFN- λ 3 did not trigger any

additive/synergistic cytokine production (**Fig. 9C**). Worthy of note is that in this group of experiments, the 42 h strong CXCL10/IP-10 producer pattern was reproduced also in response to IFN- λ 1, further corroborating the validity of our observations.

DISCUSSION

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

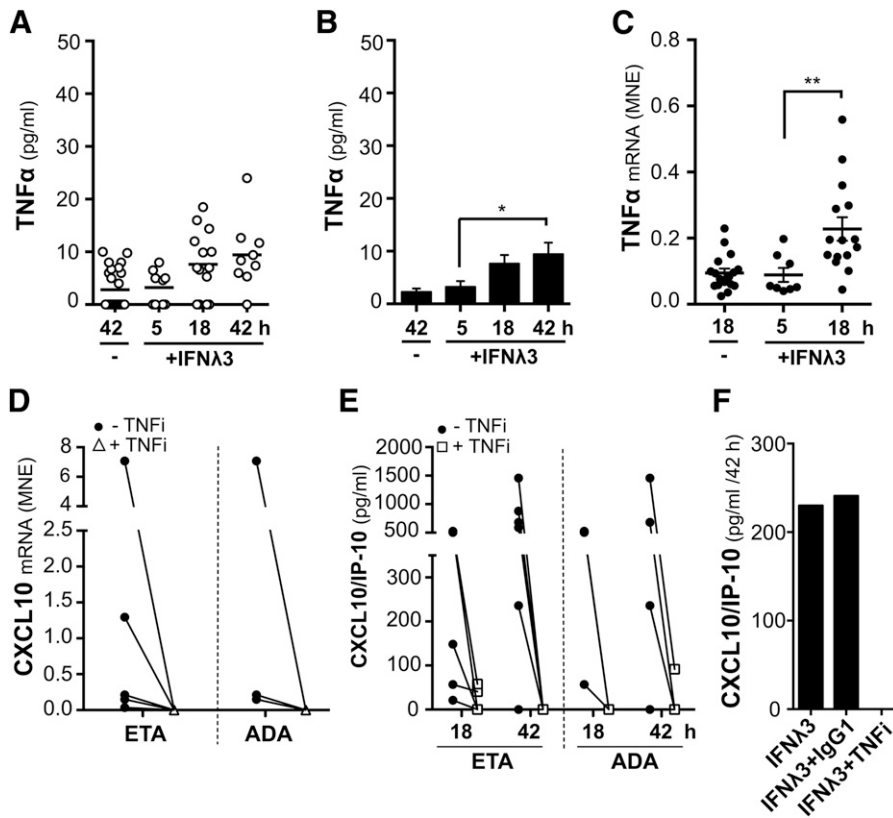


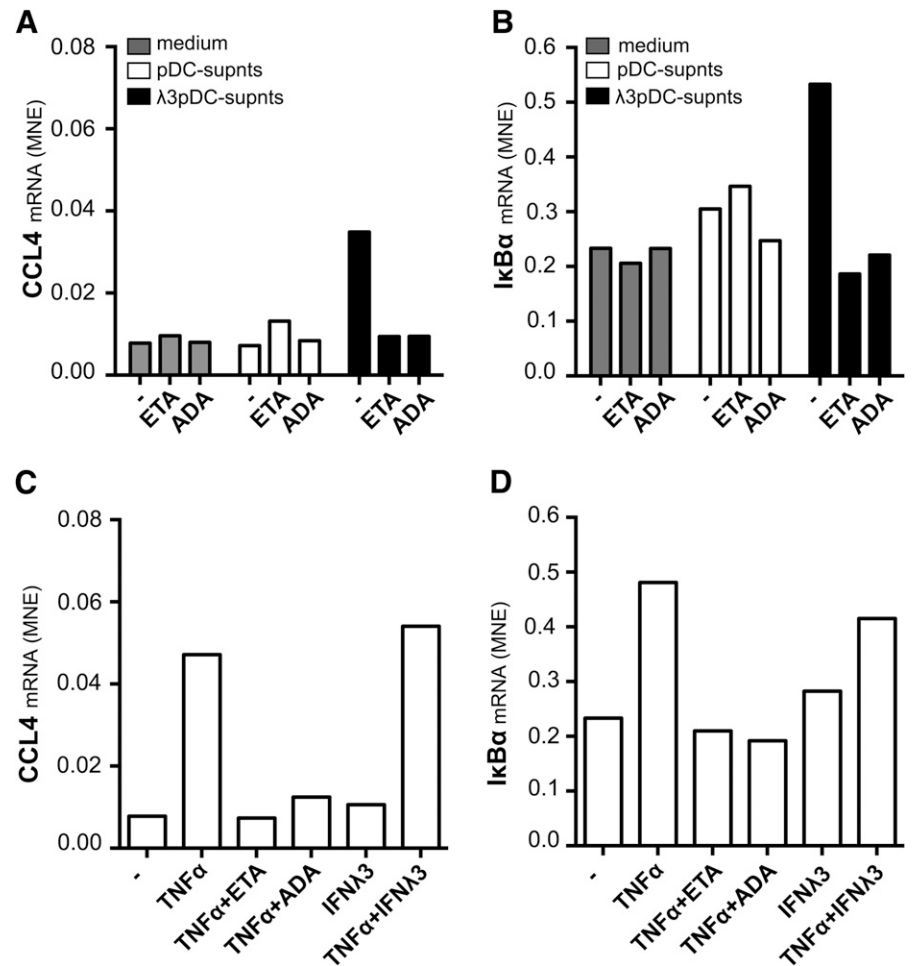
Figure 7. Role of endogenous TNF- α in mediating the induction of CXCL10/IP-10 in IFN- λ 3-treated pDCs. pDCs were incubated with or without 30 ng/ml IFN- λ 3 for up to 42 h to analyze gene expression and extracellular production of TNF- α . (A) Results of all individual measurements; (B) means \pm SEM of A ($n = 13$ – 19). (C) TNF- α mRNA expression in IFN- λ 3-treated pDCs. (D and E) pDCs were pretreated for 30 min with or without TNF- α inhibitors (TNFi), namely 5 μ g/ml ETA or 2.5 μ g/ml ADA, as well as their isotype control antibodies (human IgG₁; F) before incubation with IFN- λ 3. After 18 (D and E) and 42 h (E), CXCL10 mRNA expression (D) and production (E) were evaluated and results displayed as absolute values without or with TNFi. Isotype control antibodies for TNFi did not affect the production of CXCL10/IP-10 induced by IFN- λ 3, as illustrated by F, displaying 1 of the experiments reported in E. Significant increases: * $P < 0.05$, ** $P < 0.01$.

proapoptotic effect exerted by DEX in pDCs [17] to enhance their CCR7, CD62L, CD80, CD83, ICOS-L, and MHC-I expression levels [16, 17]; to reduce IL-10, IL-13, and IFN- γ production by cocultures of pDCs with allogenic T cells [16]; and to potentiate the production of IFN- α induced by CpG-A [18]. Interestingly, a comparison of the potency between IFN- λ 3 and IFN- λ 1, used at identical concentrations, revealed that they were essentially equivalent in terms of promotion of survival, antigen modulation, and cytokine production in pDCs and that they do not function additively/synergistically.

Notably, analysis of the patterns of IFN- α and CXCL10/IP-10 production by pDCs incubated with IFN- λ 3 uncovered a number of peculiar features. For instance, in some but not all donors, we noticed that CXCL10/IP-10 was produced by IFN- λ 3-treated pDCs before IFN- α , therefore indicating that CXCL10/IP-10 might not be necessarily dependent on IFN- α , as also reported to occur in pDCs incubated with CpG [52]. In fact, whereas maximal IFN- α yields were always detected after 42 h of pDC incubation with IFN- λ 3, peak levels of CXCL10/IP-10 were observed to occur, depending on the donor, earlier. Another observation that intrigued us was the large variability in the amounts of IFN- α and CXCL10/IP-10 detectable in supernatants harvested from IFN- λ 3-treated pDCs (ranging from a few to thousands of picograms/milliliter), which we initially considered “expectable,” given the use of primary cells. However, a more diligent, retrospective scrutiny of our data globally revealed that healthy donors could be differentiated into 3 groups based on the kinetics and the amounts of CXCL10/IP-10 produced by their IFN- λ 3-treated pDCs: 1) 1 group, including donors whom

we defined low CXCL10/IP-10 producers, whose IFN- λ 3-treated pDCs release modest quantities of CXCL10/IP-10; 2) a second group, including donors whom we called 18 h strong CXCL10/IP-10 producers, whose IFN- λ 3-treated pDCs produce remarkably elevated levels of CXCL10/IP-10 already after 18 h; 3) and a third group, including donors whom we called 42 h strong CXCL10/IP-10 producers, characterized by pDCs expressing very high CXCL10/IP-10 levels at the 42 h time point. Such patterns recall observations made in a previous study describing that PBMCs from healthy donors appeared to function as “early” or “late” responders to IFN- λ 1: early responders showed peak mRNA levels for CXCL9, CXCL10, and CXCL11 between 15 and 240 min, whereas late responders peaked between 24 and 72 h [53]. Concomitantly, 2 types of “IFN- α producers” could also be distinguishable in our study, namely those whose IFN- λ 3-treated pDCs produce IFN- α at levels <150 pg/ml and those producing the cytokine at levels >500 pg/ml. Interestingly, whereas all 42 h strong CXCL10/IP-10 producers corresponded to the donors whose pDCs produced high levels of IFN- α (>500 pg/ml), the 18 h strong CXCL10/IP-10 producers did not. Altogether, data depict a very complex scenario, implying that donor-dependent factors might likely condition, in a differential manner, the production of IFN- α and CXCL10/IP-10 by IFN- λ 3-treated pDCs. The molecular bases underlying the variable capacity of pDCs to produce IFN- α and CXCL10/IP-10 by the various donor typologies and its potential biologic implication(s) are unknown and need to be investigated better. In such regard, a number of polymorphisms are present at the level of the IFN- α [54] and CXCL10 [55–57]

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



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

Whatever the case is, experiments that used antibodies neutralizing the IFN- α R definitively confirmed that the production of CXCL10/IP-10 triggered by IFN- λ 3-treated pDCs, isolated from the 42 h strong CXCL10/IP-10 producers, is scarcely dependent on endogenous IFN- α . Even though no 18 h strong CXCL10/IP-10 producers could be included in IFN- α R-neutralizing experiments, based on the observations described above, there is no reason to believe that production of CXCL10/IP-10 by their IFN- λ 3-treated pDCs may be more dependent on endogenous IFN- α than in 42 h strong CXCL10/IP-10 producers. By contrast, the use of different TNF- α

inhibitors, namely, ADA, ETA, and infliximab [49], allowed us to uncover that the expression of CXCL10/IP-10 by IFN- λ 3-treated pDCs is totally driven by endogenously produced TNF- α , regardless of the CXCL10/IP-10 producer group. Under the same experimental conditions, endogenous TNF- α was found to contribute slightly to the production of IFN- α [unpublished results] and not to affect pDC survival, pointing to its distinctive role in promoting CXCL10/IP-10 expression. Moreover, no inhibition of CXCL10/IP-10 expression by ETA was observed in pDCs incubated with R837 (which produces massive amounts of TNF- α , IFN- α , and CXCL10/IP-10) [23, 46, 59], thus excluding a “reverse signaling”-mediated inhibitory effect [60] and indicating an apparently specific effect of endogenous TNF- α under IFN- λ 3 treatment only. Intriguingly, notwithstanding the results obtained by TNF- α blockers, exogenous TNF- α alone, at doses ranging from 0.01 to 10 ng/ml, triggered only minute amounts of CXCL10/IP-10 in pDCs, even though it induced, for instance, CXCL8 mRNA and pDC maturation [unpublished results]. On the other hand, supernatants harvested from pDCs treated with IFN- λ 3 for 18 h displayed the capacity to induce, in a TNF- α -dependent manner, the expression of CCL4 and I κ B α mRNAs in CD14 $^{+}$ monocytes, thus proving definitively that the TNF- α , present in pDC-derived supernatants, is biologically active.

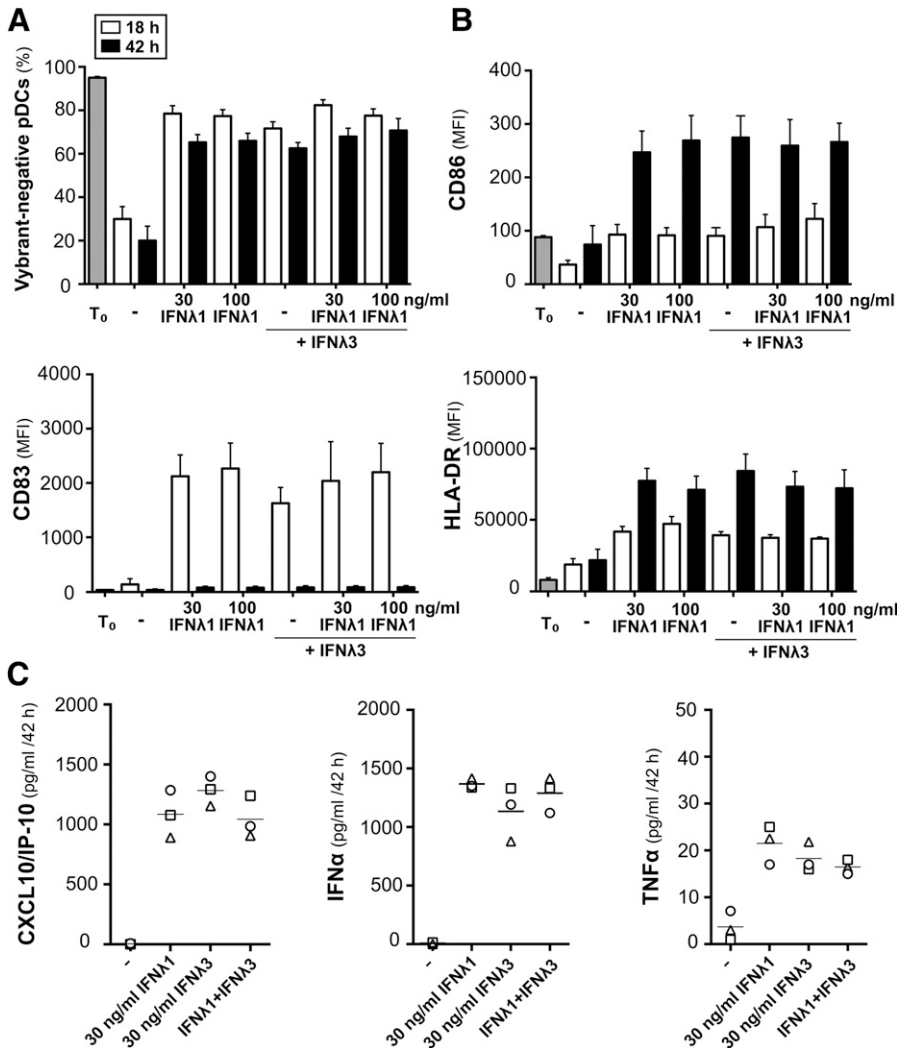


Figure 9. IFN- λ 1 and IFN- λ 3 are equally efficient in promoting survival, antigen modulation, and cytokine production in pDCs. Freshly isolated pDCs were incubated in the presence or absence of 30 ng/ml IFN- λ 3 with or without 30 or 100 ng/ml IFN- λ 1. After 18 and 42 h, pDCs were harvested and analyzed for viability (A) or HLA-DR, CD86, and CD83 expression (by use of Vybrant DyeCycle; B) by flow cytometry. For each antigen, bar graphs report the results as MFI, calculated after subtracting the MFI given by the correspondent isotype control antibodies or the basal fluorescence ($n = 3$). (C) Absolute production of CXCL10/IP-10, IFN- α , and TNF- α , measured in supernatants from pDCs incubated for 42 h. Data are from the same 3 donors reported in A and B.

A number of reasons might explain why endogenously produced TNF- α is able to control the production of CXCL10/IP-10 in pDCs incubated with IFN- λ 3. One possibility might be that the CXCL10 locus, following treatment with IFN- λ 3, but not spontaneously, undergoes a chromatin reorganization that becomes accessible to the signals triggered by endogenous TNF- α . If so, such a scenario would resemble what occurs in human neutrophils with regard to their production of IL-6 in response to R848 and/or TNF- α [61]. An alternative, more likely explanation, relies instead on the known molecular mechanisms controlling an optimal CXCL10 transcription, which is well demonstrated to involve a synergistic action of the transcription factors STAT and NF- κ B [43–45]. According to such an explanation, we would speculate that STATs and NF- κ B would be mobilized to the CXCL10 promoter in pDCs incubated with IFN- λ 3: STATs directly in response to IFN- λ 3 and NF- κ B following activation by endogenously produced TNF- α .

In conclusion, our study greatly extends our knowledge on the biologic effects that IFN- λ 3 exerts on pDCs, which might be relevant in the context of viral infections, particularly in the case of HCV progression. Accordingly, if pDCs are recruited into the

liver of chronic hepatitis C patients [62], then it is tempting to speculate that locally produced IFN- λ 3 activates pDCs to express ISG genes and to produce TNF- α , IFN- α , and CXCL10/IP-10. Locally, TNF- α may contribute to hepatic inflammation and cell death, whereas CXCL10/IP-10 and IFN- α could influence the recruitment and activation of CXCR3⁺ cells (such as monocytes, NK cells, and Th1 cells) [27] in the infected liver. Conceivably, these phenomena would all sustain the progression of inflammation and immune response, eventually leading to chronic infection. More broadly, our data also suggest that IFN- λ 3, by triggering the production of IFN- α , CXCL10/IP-10, and TNF- α by pDCs, may impact on the cytokine balance controlling the polarization/recruitment of Th cells, favoring, in turn, the Th1 phenotype, in line with what was previously shown for IFN- λ 1 and IFN- λ 2 [63, 64].

AUTHORSHIP

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

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:

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