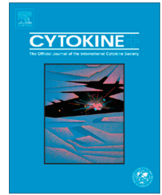
Cytokine 86 (2016) 124–131

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine



Synergistic production of TNFα and IFNα by human pDCs incubated with IFNλ3 and IL-3

Giulia Finotti, Nicola Tamassia, Marco A. Cassatella *

Department of Medicine, Section of General Pathology, University of Verona, Verona, Italy

ARTICLE INFO

Article history:
Received 10 June 2016
Received in revised form 3 August 2016
Accepted 3 August 2016

Keywords:
plasmacytoid dendritic cells
interferon lambda
IL-3
innate immunity

ABSTRACT

In this study, we investigated whether IFNλ3 and IL-3 reciprocally influence their capacity to activate various functions of human plasmacytoid dendritic cells (pDCs). In fact, we preliminarily observed that IFNλ3 upregulates the expression of the IL-3Rα (CD123), while IL-3 augments the expression of IFNλR1 in pDCs. As a result, we found that combination of IFNλ3 and IL-3 induces a strong potentiation in the production of TNFα, IFNα, as well as in the expression of Interferon-Stimulated Gene (ISG) mRNAs by pDCs, as compared to either IFNλ3 or IL-3 alone. In such regard, we found that endogenous IFNα autocrinally promotes the expression of ISG mRNAs in IL-3-, but not in IFNλ3 plus IL-3-, treated pDCs. Moreover, we uncovered that the production of IFNα by IFNλ3 plus IL-3-treated pDCs is mostly dependent on endogenously produced TNFα. Altogether, our data demonstrate that IFNλ3 and IL-3 collaborate to promote, at maximal levels, discrete functional responses of human pDCs.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Type III interferons (IFNs)/IFNλs include four members, namely IFNλ1, IFNλ2, IFNλ3 and the more recently identified IFNλ4 [1,2], that share functional similarities with type I IFNs (IFNα and IFNβ), in particular a direct anti-viral effect [1,3]. In fact, IFNλs and type I IFNs, *via* the Jak-STAT signaling pathway, trigger the transcriptional activation of hundreds of Interferon-Stimulated Genes (ISGs), which, ultimately, are responsible for the antiviral and immune IFN-mediated effect [3,4]. On the other hand, a difference between the two IFN families relies on the composition and distribution of their receptors. Accordingly, type I IFN signal through a heterodimeric receptor, composed by IFNαR1 and IFNαR2 subunits, widely distributed in most cell types, including all leukocytes subpopulations [5]. By contrast, IFNλs signal through a heterodimeric receptor complex composed of IL10R2 and a specific IFNλR1 [6,7]: while IL10R2 is ubiquitously expressed, IFNλR1 displays a restricted tissue expression that is limited to epithelial cells such hepatocytes or cells of the respiratory, gastrointestinal and reproductive tracts, as well as to discrete leukocyte populations, including plasmacytoid DCs (pDCs) and B cells [8–10].

Among IFNλR1-expressing cells, pDCs represent a unique DC subset specialized in the promotion of the early phase of antiviral immune responses, given their ability to produce high amounts of IFNα and several inflammatory cytokines and chemokines in response to viruses/viral components [11]. Recently, we reported that human pDCs incubated with IFNλ3 prolong their survival, alter the expression of CD83, CD86, CD62L and HLA-DR and produce variable amounts of IFNα, CXCL10 and TNFα [12], extending previous observations on the effects of IFNλs on pDCs [9,10]. We also observed that IFNλ3 upregulates the expression of the IL-3Rα/CD123 [12], suggesting that, by this mechanism, IFNλ3 might modulate pDC responsiveness to IL-3. The latter cytokine functions as both a hematopoietic growth factor and a regulator of cell differentiation [13], and can be secreted by activated T cells, endothelial cells and mast cells under inflammatory conditions [13–15], or even detected in the tumor microenvironment [16]. pDCs, given their constitutive expression of both CD123 and IL-3Rβ/CD131 [13,17], are known to respond to IL-3, for instance in terms of survival and differentiation into mature DCs with increased expression of MHC-I and -II and co-stimulatory molecules [17,18], as well as of production of TNFα [18,19].

Abbreviations: IL-3, interleukin 3; ADA, adalimumab; DCs, dendritic cells; ETA, etanercept; IFIT1, Interferon-Induced Protein with Tetratricopeptide Repeats 1; ISG15, ISG15 Ubiquitin-Like Modifier; IFNs, interferons; IFNλR1, interferon lambda receptor 1; ISGs, interferon stimulated genes; MNE, mean normalized expression; MX1, Myxovirus Resistance 1; PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid dendritic cells; R837, imiquimod; TNFα, tumor necrosis factor alpha; ISO, isotype; MFI, Mean Fluorescence Intensity.

* Corresponding author at: Department of Medicine, Section of General Pathology, University of Verona, Strada Le Grazie 8, Verona, Italy.

E-mail address: marco.cassatella@univr.it (M.A. Cassatella).

http://dx.doi.org/10.1016/j.cyto.2016.08.005
1043-4666/© 2016 Elsevier Ltd. All rights reserved.

Based on these premises, in this study we investigated whether IFNλ3 and IL-3 may cooperate in influencing discrete functions of human pDCs.

2. Materials and methods

2.1. Isolation and culture of pDCs

pDCs were purified from PBMCs using the BDCA-4 Diamond Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of isolated pDCs (>98%) was determined by flow cytometry analysis [12]. pDCs were then suspended in RPMI 1640 medium supplemented with 10% low-endotoxin fetal bovine serum (Sigma, Saint Louis, MO, USA) and either immediately analyzed for antigen expression, or cultured in 96-well U-bottom plates (Costar, Corning Incorporated, Corning, NY) for functional assays (0.5×10^6 pDCs in 100 μl) [12]. In the latter case, cells were incubated in the presence or the absence of optimal doses of IFNλ3 (30 ng/ml, from R&D, Minneapolis, MN, USA), and IL-3 (20 ng/ml, from Miltenyi) [12] or their combination. In selected experiments, pDCs were preincubated with 5 μg/ml αIFNAR (PBL Interferon Source, Piscataway, NJ, USA); 5 μg/ml etanercept (ENBREL®, Amgen, Thousand Oaks, CA, USA), 2.5 mg/ml adalimumab (Abbott Laboratories, Abbott Park, IL, USA) or their isotype control antibodies (Abs), before treatment with stimuli.

2.2. Flow cytometry analysis

To perform phenotypic studies, pDCs were first treated with 5% human serum and then stained for 20 min at room T, using the following mAbs: FITC anti-CD303 (Miltenyi), PE anti-IFNλR1 (BioLegend, San Diego, California, USA), PE-Cy7 anti-CD123 (BioLegend), APC-Cy7 anti-HLA-DRα (BioLegend), as well as their related isotype controls, as previously reported [12]. Sample fluorescence was then measured by using an eight-color MACSQuant Analyzer (Miltenyi), and data analysis performed by FlowJo software Version 8.8.6 (TreeStar). Phenotypic 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 was performed as previously reported [12].

2.3. Gene expression studies

Total RNA was extracted from pDCs after lysis by RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands). To completely remove any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation. Purified RNA was then reverse-transcribed into cDNA and gene expression studies performed by reverse transcription real-time PCR (RT-qPCR), as previously reported [12].

2.4. Cytokine measurement

IFNα and TNFα production was measured in pDC-derived supernatants using specific ELISA kits purchased from, respectively, Mabtech (Nacka Strand, Sweden) and eBioscience. Detection limits of these ELISAs were: 7 pg/ml for IFNα and 4 pg/ml for TNFα.

2.5. Statistical analysis

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

3. Results

3.1. Both IFNλ3 and IL-3 upregulate the expression of CD123/IL-3Rα and IFNλR1 in human pDCs

Flow cytometry experiments revealed that both IFNλ3 and IL-3, in addition to increase the levels of CD123 (Fig. 1A) [12], also upregulate the expression of IFNλR1 in human pDCs incubated for 18 h (Fig. 1B), therefore suggesting that the two cytokines may reciprocally influence responsiveness of pDCs to each other. Since we have previously shown that either IFNλ3 or IL-3 prolong the viability of pDCs [12], we investigated whether they could promote a more potent pDC prosurvival effect when used in combination. As shown in Fig. 1C, that was not the case, because each cytokine by itself was found to substantially trigger a maximal increase of pDC survival. Similarly, neither CD123 (Fig. 1A), nor

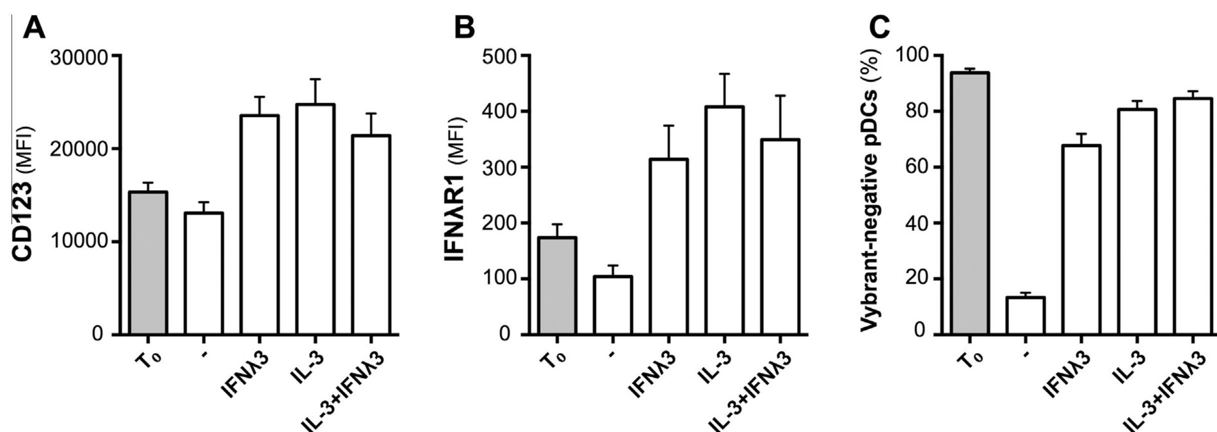


Fig. 1. Effect of IFNλ3 and/or IL-3 on the IFNλ3R1 and IL-3Rα expression, as well as the survival, by human pDCs. (A, B) pDCs were incubated with or without 30 ng/ml IFNλ3 and/or 20 ng/ml IL-3. Cells were harvested after 18 h and analyzed, by flow cytometry, for CD123 (A) and IFNλR1 (B) expression in comparison to freshly isolated pDCs (T_0). Bar graphs report the MFI means ± SEM (n = 7–15). MFI was calculated after subtracting the MFI given by the correspondent isotype control antibodies or the basal fluorescence. (C) pDCs were stained with the Vibrant DyeCycle, to assess their viability under the same experimental conditions. Gating strategy to identify live pDCs was performed as previously described [13].

IFNλR1 (Fig. 1B) expression was additively or synergistically enhanced by IFNλ3 and IL-3 used in combination.

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

Subsequently, we analyzed the extracellular production of IFNα by pDCs incubated with or without IFNλ3 and/or IL-3 for up to 18 h. As shown in Fig. 2 (panel A displaying the values of all individual donors; panel B the related means ± SEM), minimal amounts of IFNα (less than 10 pg/ml) were detected in supernatants from pDCs treated with either IFNλ3 or IL-3, but not with culture medium only. By contrast, a synergistic production of IFNα was measured in supernatants harvested from pDCs incubated with both IFNλ3 and IL-3 for 18, but not 5 h (Fig. 2A and B), concomitantly with effects displayed also at gene expression level (Fig. 2C). In these latter experiments, either IFNλ3, or IL-3, were found to modestly, but significantly, induce IFNα mRNA accumulation, at the 18 h time-point (Fig. 2C). Under the same experimental conditions, pDCs cultured with R837/imiquimod, a classic activator of these cells via TLR7 [19], produced remarkable levels of IFNα already after 5 h (390 ± 205 pg/ml, n = 3), that even increase at 18 h (1821 ± 680 pg/ml, n = 4; data not shown). Interestingly, IL-3 was found to also upregulate the mRNA expression of various ISGs, such as Interferon-Induced Protein with Tetratricopeptide Repeats 1 (IFIT1), ISG15 Ubiquitin-Like Modifier (ISG15) and Myxovirus

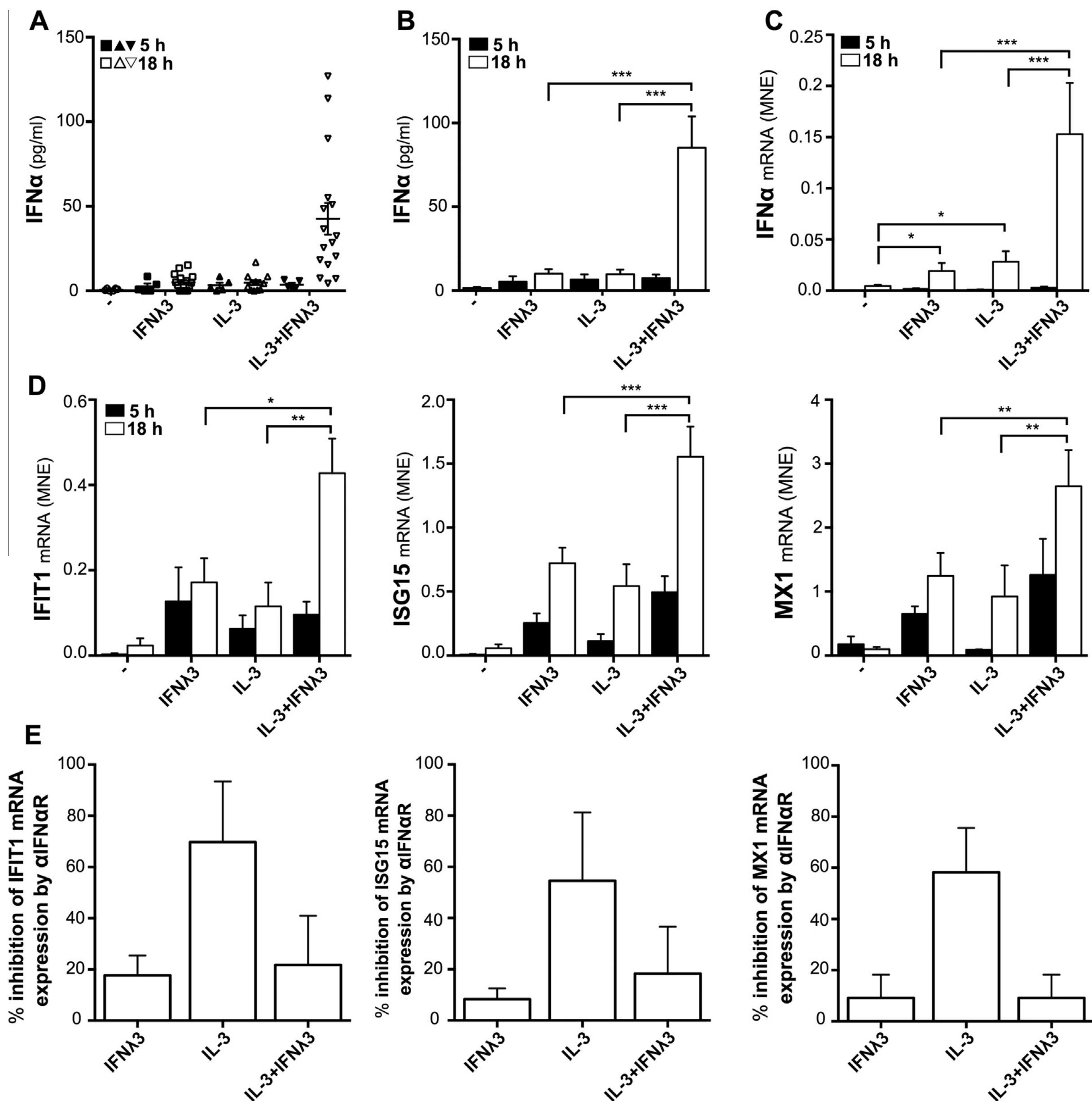


Fig. 2. Production of IFNα by pDCs incubated with IL-3 and/or IFNλ3 and involvement of endogenous IFNα in mediating ISG mRNA expression. (A, B) pDCs were incubated with or without 30 ng/ml IFNλ3 and/or 20 ng/ml IL-3 for 5 and 18 h. Cell-free supernatants were then collected and extracellular IFNα measured by ELISA. Panel (A) shows the results of all individual experiments for the displayed conditions, while panel (B) shows their related means ± SEM (n = 5–16). No IFNα was measurable in supernatants from untreated pDCs. (C, D) pDCs were incubated for 5 and 18 h and evaluated for IFNα (C), IFIT1, ISG15 and MX1 (D) mRNA expression by RT-qPCR. Results (mean ± SEM, n = 5–14) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. (E) pDCs were incubated with or without 5 μg/ml of αIFNαR or mouse IgG_{2a} (isotype control antibody, not shown) for 30 min and then incubated with IFNλ3 and/or IL-3 for 18 h. Bar graphs show the percentage of inhibition of IFIT1, ISG15 and MX1 mRNA expression exerted by αIFNαR antibodies (means ± SEM, n = 4). Under the same experimental conditions, isotype control antibodies did not affect ISG mRNA expression (data not shown). Significant variations: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Resistance 1 (MX1) in 18 h-treated pDCs, while IFNλ3 was found to trigger a similar effect already after 5 h (Fig. 2D). Notably, ISG mRNA expression was synergistically upregulated in pDCs cotreated with IFNλ3 and IL-3 at both the 5 and 18 h time-points, with the exception of IFIT1 mRNA at 5 h (Fig. 2D).

To clarify if, and to what extent, endogenous IFNα could be responsible for the induction of ISG mRNA expression in pDCs treated with IL-3 and/or IFNλ3, we performed new experiments using IFNαR neutralizing antibodies (Abs) [12]. As shown in Fig. 2E, upregulation of IFIT1, ISG15 and MX1 mRNA expression in pDCs treated with IL-3 for 18 h was largely blocked by the αIFNαR Abs, suggesting a functional autocrine action by endogenous IFNα. By contrast, ISG expression induced in pDCs either by IFNλ3 alone, or by IFNλ3 plus IL-3, was only slightly affected by the αIFNαR Abs (Fig. 2E), indicating a direct effect of IFNλ3 in triggering the expression of IFIT1, ISG15 and MX1 mRNAs. Taken together, data demonstrate that IL-3 induces the production of small, but biologicaly active amounts of IFNα from human pDCs, which in turn autocrinally promotes the expression of ISG mRNAs. Data also show that IFNλ3 plus IL-3 synergize in inducing the producton of IFNα, as well as the expression of ISG mRNA: the latter phenomenon, however, occurs independently from endogenous IFNα.

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

Measurement of TNFα in the same pDC-derived supernatants confirmed [12] that IFNλ3 induces the production of very low, but detectable, extracellular levels of the cytokine (Fig. 3A and B). Similarly, IL-3 confirmed [19] to trigger a remarkable production of TNFα by pDCs already after 5 h of incubation (Fig. 3A and B). Interestingly, the amounts of TNFα detected in supernatants from IFNλ3 plus IL-3-treated pDCs were significantly higher than those from IL-3-treated pDCs, but only at the 18 h time-point (Fig. 3A and B), consistent with findings at the TNFα mRNA level (Fig. 3C). Expression of TNFα mRNA in pDCs treated with IL-3 or IFNλ3 alone was instead maximal after 5 and 18 h, respectively (Fig. 3C). Given that endogenous IFNα was previously shown to be involved in supporting TNFα production in IL-3 plus CpG-stimulated pDCs [20], we investigated its role by incubating IFNλ3- and/or IL-3-treated pDCs in the presence of αIFNαR Abs for 18 h. As shown in Fig. 3D, the production of TNFα by IFNλ3 and/or IL-3-treated pDCs was minimally influenced by the presence of αIFNαR Abs, suggesting that in our conditions endogenously produced IFNα is substantially dispensable for the generation of TNFα, independently from its yields.

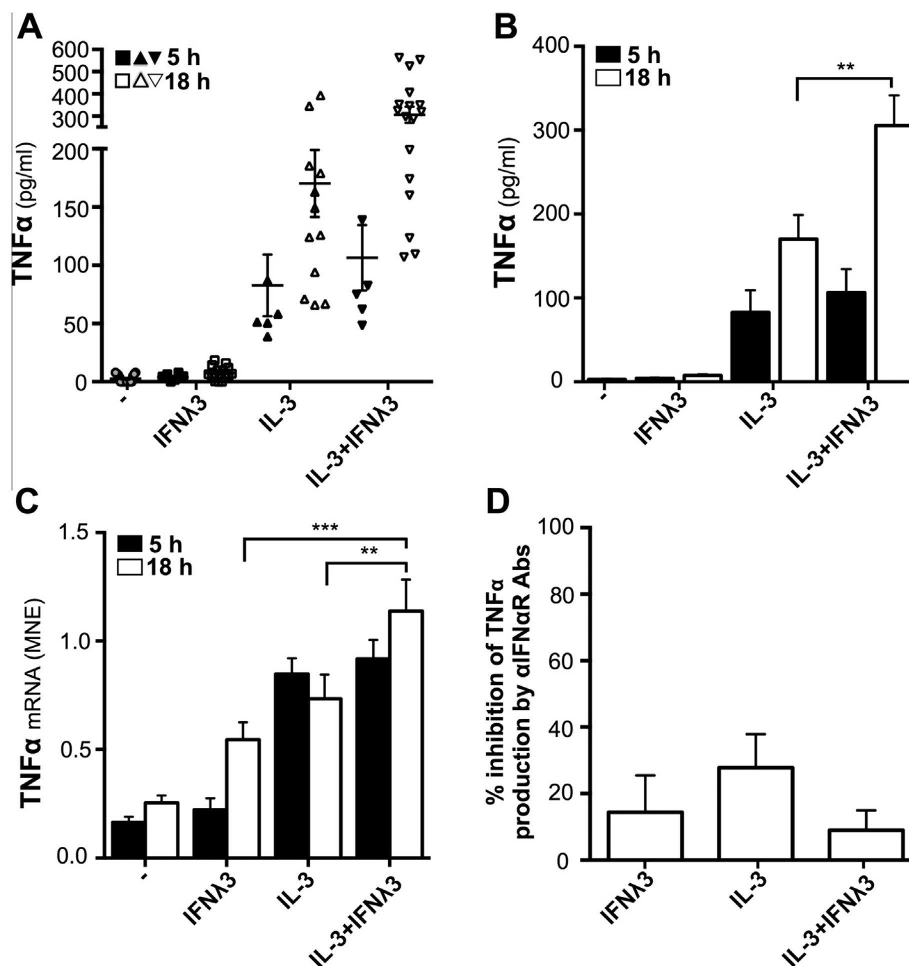


Fig. 3. Synergistic production of TNFα by pDCs incubated with IL-3 plus IFNλ3. (A–C) pDCs were incubated with or without 30 ng/ml IFNλ3 and/or 20 ng/ml IL-3 for 5 and 18 h, to analyze their TNFα production (A and B) or TNFα gene expression (panel C). (A) Results from all individual measurements by ELISA. (B) Means ± SEM of samples in A (n = 5–17). (C) TNFα mRNA expression in IFNλ3 and/or IL-3-treated pDCs. Gene expression data (mean ± SEM, n = 8–14) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization. (D) pDCs were pretreated for 30 min with or without 5 μg/ml αIFNαR or mouse IgG_{2a} (not shown) and then incubated with IFNλ3 and/or IL-3for 18 h. Bar graphs show the percentages of inhibition on TNFα production (n = 3–5) exerted by αIFNαR antibodies. Under the same experimental conditions, isotype control antibodies did not affect TNFα production (data not shown). Significant variations: $^{**}P < 0.01$; $^{***}P < 0.001$.

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

Because it has been previously shown that, in primary or synovial macrophages from patients with rheumatoid arthritis, TNFα may function as an endogenus inducer of type I IFN production [21,22], we investigated whether the same could occur under our experimental conditions. As shown in Fig. 4, etanercept (ETA), a TNFα blocker [23], potently inhibited both IFNα mRNA expression (Fig. 4A) and IFNα production (Fig. 4B) in pDCs treated with IFNλ3 plus IL-3 for 18 h. It was not possible to precisely quantify the effect of ETA on the production of IFNα by IFNλ3- or IL-3-treated pDCs due to the scarce amounts of IFNα produced. However, expression of IFNα mRNA was almost completely abrogated by ETA in pDCs treated with either IFNλ3 or IL-3 (data not shown). Nonetheless, ETA almost completely blocked IFIT1 and ISG15 (Fig. 4C) mRNA expression in pDCs treated with IFNλ3 plus IL-3, consistent with the results shown in Fig. 4A. ETA also diminished the expression of IFIT1 and ISG15 mRNA induced not only by IFNλ3, but also by IL-3 alone, in the latter case more effectively (Fig. 4C). Finally, TNFα exogenously added to pDC cultures was found to directly induce either the production of IFNα (Fig. 4D), at levels similar to IFNλ3 or IL-3 (see Fig. 2A), or a rapid expression of ISG15 mRNA (Fig. 4E). Altogether, data demonstrate that the synergistic production of IFNα by pDCs incubated with IFNλ3 plus IL-3 is mainly mediated by endogenous TNFα, which is synergistically induced by the two stimuli used in combination.

4. Discussion

In this study, we investigated whether IFNλ3 and IL-3 reciprocally influence their capacity to activate various functions of human pDCs, given that IFNλ3 increases the expression of IL-3Rα/CD123 [12], while IL-3 augments the expression of IFNλR1. Accordingly, we found that pDCs incubated for 18 h with IFNλ3 plus IL-3 produce IFNα at levels synergistically higher than pDCs treated with either IFNλ3 or IL-3 alone. Even though we further show that IFNα mRNA expression substantially correlates with IFNα production, we did not investigate the intracellular signaling pathways whereby IFNλ3 plus IL-3 synergistically trigger the transcription of IFNα. In such regards, it might be possible that IFNλ3 positively regulates the pathways triggered in IL-3-treated pDCs and involved in IFNα production, namely the PI3K- and MAPK-signaling cascades [24,25]. It cannot be excluded that the two cytokines might act either at the post-transcriptional level, for instance prolonging IFNα mRNA stability or amplifying IFNα mRNA translation, or at the chromatin level, favoring an increase accessibility of the transcriptional machinery at the IFNα locus. Whatever the case is, since both IFNλ3 and IL-3 alone were found to actively induce IFNα mRNA expression and production in pDCs, we would favor the notion that the different signaling pathways triggered by the two cytokines ultimately converge to synergistically increase IFNα gene transcription and protein production.

Since we uncovered that the mRNA expression of ISGs, including IFIT1, ISG15 and MX1 was also synergistically increased in pDCs treated with IFNλ3 plus IL-3, we asked whether

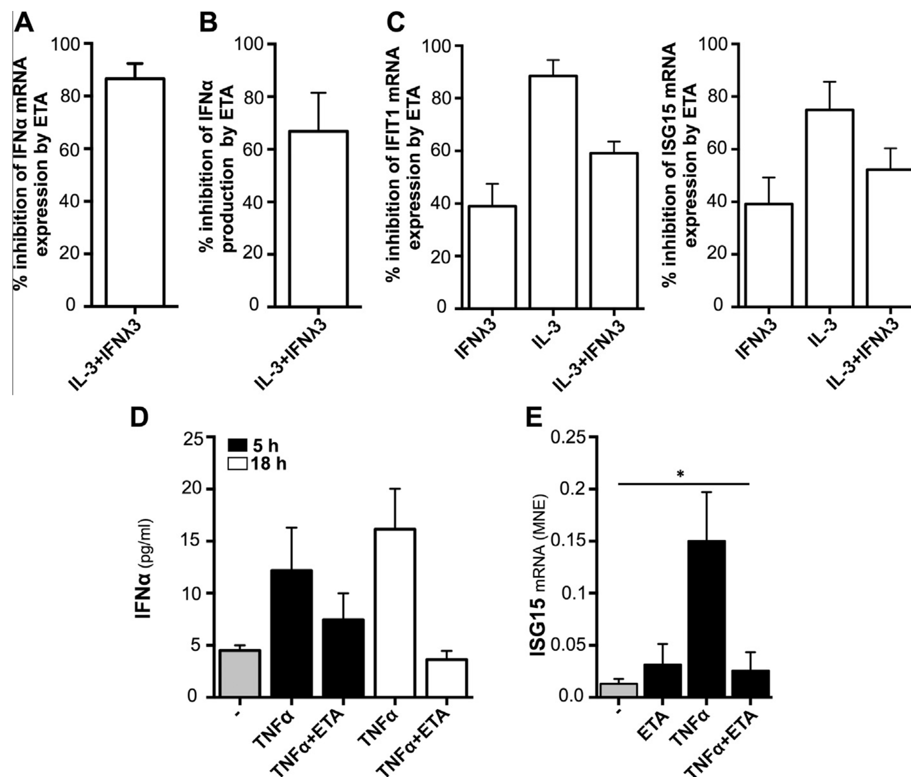


Fig. 4. Role of endogenous TNFα in mediating the production of IFNα and the expression of ISG mRNAs in pDCs treated with IL-3 plus IFNλ3. (A, B) pDCs were pretreated with or without 5 μg/ml ETA or human IgG_1 (isotype control antibodies) for 30 min, and then incubated with IL-3 plus IFNλ3 for 18 h. Bar graphs shows the percentage of inhibition on IFNα mRNA expression (A) and IFNα production (B) (means ± SEM, n = 3–4) exerted by ETA. Under the same experimental conditions, isotype control antibodies did not affect IFNα production or mRNA expression (data not shown). (C) pDCs were pretreated with or without 5 μg/ml ETA and then incubated with IFNλ3 and/or IL-3 for 18 h. The percentage of inhibition on IFIT1 and ISG15 mRNA expression is reported (means ± SEM, n = 3). (D) pDCs were cultured in the presence or absence of 10 ng/ml TNFα, for 5 and 18 h, to measure their capacity to produce IFNα (values expressed as mean ± SEM, n = 4). (E) IFIT1 mRNA expression in pDCs treated with 10 ng/ml TNFα for 3 h. Gene expression data (mean ± SEM, n = 4) are depicted as mean normalized expression (MNE) units after RPL32 mRNA normalization.

endogenously produced IFNα could be responsible for this phenomenon. Unexpectedly, IFNαR neutralizing antibodies only slightly decreased ISG mRNA expression in IFNλ3 plus IL-3-treated pDCs. Such a minor role of endogenous IFNα in inducing ISG mRNAs could be explained by the fact that it is IFNλ3 itself that directly upregulates ISG genes, given that IFNλ3 triggers signaling pathways similar to those activated by type I IFN [4]. Accordingly, IFNαR blocking experiments did not influence ISG mRNA expression observed in pDCs incubated with IFNλ3 alone. By contrast, we found that, just by itself, IL-3 upregulates in pDCs ISG mRNA expression at levels comparable to those induced by IFNλ3 alone, and, surprisingly, in a fashion totally dependent on endogenous IFNα. Altogether, data suggest that the synergistic expression of ISG mRNAs in pDCs incubated with IFNλ3 plus IL-3 derives, in part, from the a direct effect of IFNλ3, and, in part, from an action triggered by IL-3 and mediated *via* endogenous IFNα.

It has been previously shown that pDCs incubated with IL-3 produce variable amounts of TNFα [18,19]. Moreover, pDCs incubated with RNA-containing immune complexes (IC) in the presence of GM-CSF, a cytokine that shares with IL-3 the β-chain receptor for signaling, were found to produce synergistic amounts of both IFNα and TNFα as compared to RNA-IC alone [15]. Furthermore, we previously found that even IFNλ3 induces the production of low, but biologically active, levels of TNFα by human pDCs [12]. All these observations prompted us to subsequently analyze the production of TNFα by pDCs treated with IFNλ3 and/or IL-3. As a result, we found that IFNλ3 plus IL-3 induce a synergistic induction of both TNFα mRNA expression and TNFα production as compared to those induced by IL-3/IFNλ3 alone, which was detectable after 18 h and maintained up to 42 h of culture (our unpublished observations). In such regard, it is well known that the induction of TNFα mRNA expression usually depends on stimuli able to activate NFκB- and/or MAPK-dependent pathways [26,27], IL-3 being one of them [13]. Similarly, and as already proved for type I IFNs [28], also IFNλ3 is able to activate NFκB- and/or MAPK-dependent signaling pathways [29,30]. Thus, to explain how the synergistic production of TNFα by IFNλ3 plus IL-3-treated pDCs occurs at molecular/biochemical levels, we would speculate that IL-3 is mostly responsible to activate NF-κB/MAPK pathways, while IFNλ3 simply potentiates such IL-3-triggered signaling, in turn leading to a stronger TNFα mRNA expression and protein production. However, this speculation needs to be formally ascertained.

Subsequently, we explored whether, in IL-3-treated pDCs, endogenous IFNα could have some role in inducing TNFα production, similarly to its action on ISG mRNA expression. As putative control, we used pDCs treated with IFNλ3 plus IL-3 as, under this condition, IFNα production is synergistically increased. However, IFNαR neutralizing antibodies had only a minor, or even no, effect on the production of TNFα secreted by pDCs treated either by IL-3 alone, or by IFNλ3 plus IL-3, suggesting that, independently of its yields, endogenous IFNα is not required for the production of TNFα. Conversely, experiments using different TNFα inhibitors, namely etanercept and adalimumab (our unpublished observations) allowed us to uncover that the production of IFNα by IFNλ3 plus IL-3-treated pDCs is mostly driven by endogenous TNFα. Similarly, we found that also the expression of ISGs in IFNλ3 plus IL-3-treated pDCs is mostly driven by endogenous TNFα. Thus, data suggest that, in IFNλ3 plus IL-3-stimulated pDCs, endogenous TNFα is responsible for both the synergistic production of IFNα and ISG mRNA expression. Interestingly, we found that ETA almost completely abrogates the expression of both IFNα and ISG mRNAs even in pDCs treated with either IFNλ3 or IL-3. However, it was not possible to precisely quantify the effect of TNFα inhibitors on the production of IFNα by either IFNλ3-, or IL-3-treated pDCs due to the scarce cytokine levels. In any case, ETA almost completely blocked IFIT1, ISG15 and MX1 mRNA expression in pDCs treated with IL-3, consistent with an inhibition on IFNα-dependent effects. A downmodulation of ISG expression by ETA was detected also in IFNλ3-treated pDCs, yet an effect IFNα-independent as, under the same experimental conditions, αIFNαR antibodies were ineffective. Although these results need to be carefully interpreted, it is possible that endogenous TNFα directly contributes to the transcriptional control of ISGs *via* NF-κB- and/or MAPK-dependent pathways, as also proposed by the literature [31–33]. Moreover, exogenous TNFα was found to directly induce both the production of IFNα (in low amounts) and the expression of ISG15 mRNA in pDCs, confirming their responsiveness to TNFα.

In sum, in this work we report that pDCs incubated with IFNλ3 plus IL-3 synergize in triggering the production of remarkable amounts of both IFNα and TNFα. We also demonstrate that the production of IFNα by IFNλ3 plus IL-3-treated pDCs is mostly dependent on endogenously produced TNFα. Along with a direct effect of IFNλ3, endogenously produced TNFα also controls ISG mRNA expression, either directly, or via IFNα. These results are consistent with a crucial role of pDC-derived TNFα in autocrinally amplifying the production of cytokines, as previously reported in the case of CXCL10 production by pDCs [12]. Moreover, data suggest that IFNλ3 and IL-3 collaborate to promote discrete functional responses of human pDCs at maximal levels. Under this perspective, data point for an important role of IL-3, likely secreted by infiltrating T cells at the sites of infection [13,15], in enhancing pDC responses. Moreover, even IL-3 produced by endothelial cells [34] might have a role in potentiating or influencing pDC activities during their transmigration into the site of infection, lymph nodes and/or tumors [35,36]. Interestingly, it has been recently shown that, during sepsis, a clinically serious systemic inflammation in which type I IFNs play critical roles [37], IL-3 is produced in the spleen, mostly by activated B and T lymphocytes [38]. In this context, IFNα/λ-enhanced B cell activation results in increased cytokine and Ig production during TLR7 challenge [39]. In addition, a strict pDC-B cell crosstalk exists as B cells enhance IFNα, and possibly IFNλs, production by pDCs via cell–cell contact or soluble factors [40]. For these reasons, it is plausible that, not only in sepsis, but also in other autoimmune diseases, B cells could promote and amplify, via their production of IL-3, the activation and function of pDCs. A potential collaboration between IFNλ3 and IL-3 may even occur in tumors in which pDCs, IFNλs and also IL-3 are detectable [3,16,36,41]. Accordingly, IL-3 and/or IFNλ3 could favor pDC antitumoral activities [11].

Finally, it is worth mentioning that IL-3 is often used to maintain pDC survival in culture [20,42–44], particularly in experiments in which researchers investigate the effect of a given stimulus on pDCs. However, the potential contribution of IL-3 in influencing the final results is often ignored [43,45]. Our data, highlighting that IL-3 may induce a number of responses that might sum up to those exerted by the stimulus under investigation (in this case IFNλ3), suggest that the potential contribution of IL-3 need to be always taken into consideration for a correct interpretation of the final results.

Authorship

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

Disclosures

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro – Italy (AIRC, IG-15454) to M.A.C. G.F. is recipient of a triennial fellowship from Fondazione Italiana per la Ricerca sul Cancro (FIRC).

References

[1] A. Egli, D.M. Santer, D. O'Shea, D.L. Tyrrell, M. Houghton, The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections, Emerg. Microbes Infect. 3 (2014) 51, http://dx.doi.org/10.1038/emi.2014.51.

[2] S.M. Laidlaw, L.B. Dustin, Interferon lambda: opportunities, risks, and uncertainties in the fight against HCV, Front. Immunol. 5 (2014), http://dx.doi.org/10.3389/fimmu.2014.00545.

[3] H. Lazear, T. Nice, M. Diamond, Interferon-λ: immune functions at barrier surfaces and beyond, Immunity 43 (2015) 15–28, http://dx.doi.org/10.1016/j.immuni.2015.07.001.

[4] R.P. Donnelly, S.V. Kotenko, Interferon-lambda: a new addition to an old family, J. Interferon Cytokine Res. 30 (2010) 555–564, http://dx.doi.org/10.1089/jir.2010.0078.

[5] N.A. de Weerd, T. Nguyen, The interferons and their receptors-distribution and regulation, Immunol. Cell Biol. (2012) 1–9, http://dx.doi.org/10.1038/icb.2012.9.

[6] P. Hermant, T. Michiels, Interferon-λ in the context of viral infections: production, response and therapeutic implications, J. Innate Immun. (2014) 563–574, http://dx.doi.org/10.1159/000360084.

[7] P. Sheppard, W. Kindsvogel, W. Xu, K. Henderson, S. Schlutsmeyer, T.E. Whitmore, R. Kuestner, U. Garrigues, C. Birks, J. Roraback, C. Ostrander, D. Dong, J. Shin, S. Presnell, B. Fox, B. Haldeman, E. Cooper, D. Taft, T. Gilbert, F.J. Grant, M. Tackett, W. Krivan, G. McKnight, C. Clegg, D. Foster, K.M. Klucher, IL-28, IL-29 and their class II cytokine receptor IL-28R, Nat. Immunol. 4 (2003) 63–68, http://dx.doi.org/10.1038/ni873.

[8] C. Sommereyns, S. Paul, P. Staeheli, T. Michiels, IFN-lambda (IFN-λ) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo, PLoS Pathog. 4 (2008), http://dx.doi.org/10.1371/journal.ppat.1000017.

[9] N.J. Megjugorac, G.E. Gallagher, G. Gallagher, Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29), J. Leukoc. Biol. 86 (2009) 1359–1363, http://dx.doi.org/10.1189/jlb.0509347.

[10] Z. Yin, J. Dai, J. Deng, F. Sheikh, M. Natalia, T. Shih, A. Lewis-Antes, S.B. Amrute, U. Garrigues, S. Doyle, R.P. Donnelly, S.V. Kotenko, P. Fitzgerald-Bocarsly, Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells, J. Immunol. 189 (2012) 2735–2745, http://dx.doi.org/10.4049/jimmunol.1102038.

[11] M. Swiecki, M. Colonna, The multifaceted biology of plasmacytoid dendritic cells, Nat. Rev. Immunol. 15 (2015) 471–485, http://dx.doi.org/10.1038/nri3865.

[12] G. Finotti, N. Tamassia, F. Calzetti, G. Fattovich, M.A. Cassatella, Endogenously produced TNF-α contributes to the expression of CXCL10/IP-10 in IFN-λ3-activated plasmacytoid dendritic cells, J. Leukoc. Biol. 99 (2016) 107–119, http://dx.doi.org/10.1189/jlb.3VMA0415-144R.

[13] T.R. Hercus, U. Dhagat, W.L.T. Kan, S.E. Broughton, T.L. Nero, M. Perugini, J.J. Sandow, R.J. D'Andrea, P.G. Ekert, T. Hughes, M.W. Parker, A.F. Lopez, Signalling by the βc family of cytokines, Cytokine Growth Factor Rev. 24 (2013) 189–201, http://dx.doi.org/10.1016/j.cytogfr.2013.03.002.

[14] E.I. Korpelainen, J.R. Gamble, M.A. Vadas, A.F. Lopez, IL-3 receptor expression, regulation and function in cells of the vasculature, Immunol. Cell Biol. 74 (1996) 1–7, http://dx.doi.org/10.1038/icb.1996.1.

[15] D. Leonard, M.-L. Eloranta, N. Hagberg, O. Berggren, K. Tandre, G. Alm, L. Rönnblom, Activated T cells enhance interferon-α production by plasmacytoid dendritic cells stimulated with RNA-containing immune complexes, Ann. Rheum. Dis. (2015), http://dx.doi.org/10.1136/annrheumdis-2015-208055. annrheumdis–2015–208055.

[16] P. Dentelli, a. Rosso, C. Olgasi, G. Camussi, M.F. Brizzi, IL-3 is a novel target to interfere with tumor vasculature, Oncogene 30 (2011) 4930–4940, http://dx.doi.org/10.1038/onc.2011.204.

[17] G. Grouard, M.C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, Y.J. Liu, The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand, J. Exp. Med. 185 (1997) 1101–1111, http://dx.doi.org/10.1084/jem.185.6.1101.

[18] N. Kohrgruber, N. Halanek, M. Gröger, D. Winter, K. Rappersberger, M. Schmitt-Egenolf, G. Stingl, D. Maurer, Survival, maturation, and function of CD11c- and CD11c+ peripheral blood dendritic cells are differentially regulated by cytokines, J. Immunol. 163 (1999) 3250–3259.

[19] S.J. Gibson, J.M. Lindh, T.R. Riter, R.M. Gleason, L.M. Rogers, A.E. Fuller, J.L. Oesterich, K.B. Gorden, X. Qiu, S.W. McKane, R.J. Noelle, R.L. Miller, R.M. Kedl, P. Fitzgerald-Bocarsly, M.A. Tomai, J.P. Vasilakos, Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod, Cell. Immunol. 218 (2002) 74–86, http://dx.doi.org/10.1016/S0008-8749(02)00517-8.

[20] X. Ju, M. Zenke, D.N.J. Hart, G.J. Clark, CD300a/c regulate type i interferon and TNF-α secretion by human plasmacytoid dendritic cells stimulated with TLR7 and TLR9 ligands, Blood 112 (2008) 1184–1194, http://dx.doi.org/10.1182/blood-2007-12-127951.

[21] A. Yarilina, K.-H. Park-Min, T. Antoniv, X. Hu, L.B. Ivashkiv, TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes, Nat. Immunol. 9 (2008) 378–387, http://dx.doi.org/10.1038/ni1576.

[22] R.A. Gordon, G. Grigoriev, A. Lee, G.D. Kalliolias, L.B. Ivashkiv, The interferon signature and STAT1 expression in rheumatoid arthritis synovial fluid macrophages are induced by tumor necrosis factor α and counter-regulated by the synovial fluid microenvironment, Arthritis Rheum. 64 (2012) 3119–3128, http://dx.doi.org/10.1002/art.34544.

[23] D. Tracey, L. Klareskog, E.H. Sasso, J.G. Salfeld, P.P. Tak, Tumor necrosis factor antagonist mechanisms of action: a comprehensive review, Pharmacol. Ther. 117 (2008) 244–279, http://dx.doi.org/10.1016/j.pharmthera.2007.10.001.

[24] R. Takauji, S. Iho, H. Takatsuka, S. Yamamoto, T. Takahashi, H. Kitagawa, H. Iwasaki, R. Iida, T. Yokochi, T. Matsuki, CpG-DNA-induced IFN-alpha production involves p38 MAPK-dependent STAT1 phosphorylation in human plasmacytoid dendritic cell precursors, J. Leukoc. Biol. 72 (2002) 1011–1019.

[25] C. Guiducci, C. Ghirelli, M.-A. Marloie-Provost, T. Matray, R.L. Coffman, Y.-J. Liu, F.J. Barrat, V. Soumelis, PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation, J. Exp. Med. 205 (2008) 315–322, http://dx.doi.org/10.1084/jem.20070763.

[26] N.S. Trede, A.V. Tsytsykova, T. Chatila, A.E. Goldfeld, R.S. Geha, Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B, J. Immunol. 155 (1995) 902–908.

[27] J.C. Lee, J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Landvatter, et al., A protein kinase involved in the regulation of inflammatory cytokine biosynthesis, Nature 372 (1994) 739–7346, http://dx.doi.org/10.1038/372739a0.

[28] Z. Du, L. Wei, A. Murti, S.R. Pfeffer, M. Fan, C.H. Yang, L.M. Pfeffer, Non-conventional signal transduction by type 1 interferons: the NF-kappaB pathway, J. Cell. Biochem. 102 (2007) 1087–1094, http://dx.doi.org/10.1002/jcb.21535.

[29] Z. Zhou, O.J. Hamming, N. Ank, S.R. Paludan, A.L. Nielsen, R. Hartmann, Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases, J. Virol. 81 (2007) 7749–7758, http://dx.doi.org/10.1128/JVI.02438-06.

[30] S. Brand, F. Beigel, T. Olszak, K. Zitzmann, S.T. Eichhorst, J.-M. Otte, J. Diebold, H. Diepolder, B. Adler, C.J. Auernhammer, B. Göke, J. Dambacher, IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression, Am. J. Physiol. Gastrointest. Liver Physiol. 289 (2005) G960–G968, http://dx.doi.org/10.1152/ajpgi.00126.2005.

[31] L.M. Pfeffer, J.G. Kim, S.R. Pfeffer, D.J. Carrigan, D.P. Baker, L. Weill, R. Homayouni, Role of nuclear factor-κB in the antiviral action of interferon and interferon-regulated gene expression, J. Biol. Chem. 279 (2004) 31304–31311, http://dx.doi.org/10.1074/jbc.M308975200.

[32] Y. Osawa, S. Iho, R. Takauji, H. Takatsuka, S. Yamamoto, T. Takahashi, S. Horiguchi, Y. Urasaki, T. Matsuki, S. Fujieda, Collaborative action of NF-kappaB and p38 MAPK is involved in CpG DNA-induced IFN-alpha and chemokine production in human plasmacytoid dendritic cells, J. Immunol. 177 (2006) 4841–4852, http://dx.doi.org/10.4049/jimmunol.177.7.4841.

[33] A. Forero, P.S. Moore, S.N. Sarkar, Role of IRF4 in IFN-stimulated gene induction and maintenance of Kaposi sarcoma-associated herpesvirus latency in primary effusion lymphoma cells, J. Immunol. 191 (2013) 1476–1485, http://dx.doi.org/10.4049/jimmunol.1202514.

[34] E.M. Nilsen, F.E. Johansen, F.L. Jahnsen, K.E. Lundin, T. Scholz, P. Brandtzaeg, G. Haraldsen, Cytokine profiles of cultured microvascular endothelial cells from the human intestine, Gut 42 (1998) 635–642, http://dx.doi.org/10.1136/gut.42.5.635.

[35] S. Sozzani, W. Vermi, A. Del Prete, F. Facchetti, Trafficking properties of plasmacytoid dendritic cells in health and disease, Trends Immunol. 31 (2010) 270–277, http://dx.doi.org/10.1016/j.it.2010.05.004.

[36] W. Vermi, M. Soncini, L. Melocchi, S. Sozzani, F. Facchetti, Plasmacytoid dendritic cells and cancer, J. Leukoc. Biol. 90 (2011) 681–690, http://dx.doi.org/10.1189/jlb.0411190.

[37] T. Mahieu, C. Libert, Should we inhibit type I interferons in sepsis?, Infect Immun. 75 (2007) 22–29, http://dx.doi.org/10.1128/IAI.00829-06.

[38] G.F. Weber, B.G. Chousterman, S. He, A.M. Fenn, M. Nairz, A. Anzai, T. Brenner, F. Uhle, Y. Iwamoto, C.S. Robbins, L. Noiret, S.L. Maier, T. Zönnchen, N.N. Rahbari, S. Schölch, A. Klotzsche-von Ameln, T. Chavakis, J. Weitz, S. Hofer, M. A. Weigand, M. Nahrendorf, R. Weissleder, F.K. Swirski, Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis, Science 347 (2015) 1260–1265, http://dx.doi.org/10.1126/science.aaa4268.

[39] R.a. de Groen, Z.M.a. Groothuismink, B.-S. Liu, A. Boonstra, IFN-λ is able to augment TLR-mediated activation and subsequent function of primary human B cells, J. Leukoc. Biol. 98 (2015) 623–630, http://dx.doi.org/10.1189/jlb.3A0215-041RR.

[40] O. Berggren, N. Hagberg, G. Weber, G.V. Alm, L. Rönnblom, M.-L. Eloranta, B Lymphocytes enhance interferon-alpha production by plasmacytoid dendritic cells, ARTHRITIS Rheum. 64 (2012) 3409–3419, http://dx.doi.org/10.1002/art.34599.

[41] A. Lasfar, W. Abushahba, M. Balan, K.A. Cohen-Solal, Interferon lambda: a new sword in cancer immunotherapy, Clin. Dev. Immunol. (2011), http://dx.doi.org/10.1155/2011/349575.

[42] F. Meyer-Wentrup, D. Benitez-Ribas, P.J. Tacken, C.J.A. Punt, C.G. Figdor, I.J.M. De Vries, G.J. Adema, Targeting DCIR on human plasmacytoid dendritic cells results in antigen presentation and inhibits IFN-α production, Blood 111 (2008) 4245–4253, http://dx.doi.org/10.1182/blood-2007-03-081398.

[43] S. Kim, V. Kaiser, E. Beier, M. Bechheim, M. Guenthner-Biller, A. Ablasser, M. Berger, S. Endres, G. Hartmann, V. Hornung, Self-priming determines high type I IFN production by plasmacytoid dendritic cells, Eur. J. Immunol. 44 (2014) 807–818, http://dx.doi.org/10.1002/eji.201343806.

[44] N. Bendriss-Vermare, S. Burg, H. Kanzler, L. Chaperot, T. Duhen, O. de Bouteiller, M. D'agostini, J.M. Bridon, I. Durand, J.M. Sederstrom, W. Chen, J. Plumas, M.C. Jacob, Y.J. Liu, P. Garrone, G. Trinchieri, C. Caux, F. Brière, Virus overrides the propensity of human CD40L-activated plasmacytoid dendritic cells to produce Th2 mediators through synergistic induction of IFN-γ and Th1 chemokine production, J. Leukoc. Biol. 78 (2005) 954–966, http://dx.doi.org/10.1189/jlb.0704383.

[45] P.S. Jaehn, K.S. Zaenker, J. Schmitz, A. Dzionek, Functional dichotomy of plasmacytoid dendritic cells: Antigen-specific activation of T cells versus production of type I interferon, Eur. J. Immunol. 38 (2008) 1822–1832, http://dx.doi.org/10.1002/eji.200737552.