Differential responsiveness to IFN-α and IFN-β of human mature DC through modulation of IFNAR expression

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Abstract: In human monocyte-derived dendritic cells (DC), infection with *Mycobacterium tuberculosis* and viruses or stimulation with Toll-like receptor type 3 and 4 agonists causes the release of type I interferon (IFN). Here, we describe that the IFN-β released upon stimulation with lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C) is responsible for a rapid and sustained signal transducer and activator of transcription 1 and 2 activation and expression of IFN-stimulated genes, such as the transcription factor IFN regulatory factor 7 and the chemokine CXC chemokine ligand 10. The autocrine production of IFN-β from LPS and poly I:C-matured DC (mDC) induced a temporary saturation of the response to type I IFN and a marked decline in the level of the two IFN receptor (IFNAR) subunits. It is interesting that we found that upon clearing of the released cytokines, LPS-stimulated DC reacquired full responsiveness to IFN-β but only partial responsiveness to IFN-α, and their maturation process was unaffected. Monitoring of surface and total levels of the receptor subunits showed that maximal expression of IFNAR2 resumed within 24 h of clearing, and IFNAR1 expression remained low. Thus, mDC can modulate their sensitivity to two IFN subtypes through a differential regulation of the IFNAR subunits. *J. Leukoc. Biol.* 79: 1286–1294; 2006.

Key Words: *type I IFN · cytokine receptor · TLR · LPS*

INTRODUCTION

Dendritic cells (DC) play a critical role in initiating and modulating the immune responses elicited upon recognition of infectious agents. Indeed, DC respond to microbes by triggering a complex maturation program, which results in their migration from tissue to the draining lymph nodes and in an enhanced T cell stimulatory capacity [1]. During the maturation process and in a finely regulated manner, DC produce several cytokines and chemokines that act sequentially in different microenvironments and on different leukocyte populations [2, 3]. The cytokines and chemokines produced by maturing DC (mDC) immediately after contacting the pathogens may in turn regulate, in an autocrine and paracrine manner, the production of other soluble mediators critical for the establishment of an inflammatory and innate immune response and for the recruitment of monocytes, macrophages, DC, and neutrophils. In particular, it has been demonstrated that type I interferons (IFNs) regulate the production of CX chemokine ligand 10 (CXCL10) [4, 5] as well as members of the interleukin (IL)-12 family [6–8].

We and others [9–12] demonstrated the production of type I IFNs in DC following bacterial infections and Toll-like receptor (TLR) triggering. This autocrine IFN may have critical effects on the biology of DC. IFN has been shown to promote the differentiation of human blood monocytes into DC with potent T cell stimulatory activities [13, 14] and to contribute to DC maturation [15, 16].

All IFN subtypes (IFN-β and 13 IFN-α) exert their pleiotropic activities via a heterodimeric receptor containing IFN-α/β receptor (IFNAR)1 and IFNAR2, both members of the cytokine receptor superfamily [17]. IFN, binding to the receptor complex, leads to catalytic activation of the associated tyrosine kinase 2 (Tyk2) and Janus tyrosine kinase 1 tyrosine kinases, which in turn phosphorylate signal transducer and activator of transcription (STAT)-1 and STAT-2 in most cell types [18], although activation of STAT-3, -4, -5, and -6 has also been reported [19–22]. Upon activation, STATs bind to STAT-binding elements or to IFN-stimulated response elements in the promoter of IFN-sensitive genes (ISGs). It is interesting that several primary response genes are themselves transcription factors, such as IFN regulatory factor (IRF)-1 and IRF-7, required for the induction of secondary effectors of the cellular response to IFNs and to other cytokines [23]. In addition to these mechanisms that strengthen the cellular response to IFN, negative-feedback mechanisms limit the duration and intensity of the induced signals, resulting in a selective decrease of the response to type I IFNs [24, 25].

Although DC represent one of the major sources of type I IFN and are also key responders, only a few studies have

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addressed the question of their responsiveness at different stages of maturation [26, 27]. Here, we have compared signaling responses to two IFN subtypes, IFN-α2 and IFN-β, in human immature DC (iDC) and mDC. We found that in cells stimulated with lippolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C), the released IFN-β accounts for the activation of STATs and the expression of ISGs, which cannot be enhanced further by exogenously added IFN-α2 or -β. It is interesting that upon clearing of the released IFNs through extensive washes, DC recovered full responsiveness to IFN-β but not to IFN-α. The desensitization to IFN-α correlated with a poor expression of the IFNAR1 receptor subunit. Thus, upon maturation induced by TLR-4 stimulation, DC selectively modulate their responsiveness to type I IFNs by differential expression of the receptor subunits, providing a further insight into the complexity and plasticity of the immunoregulatory response to type I IFN subtypes.

MATERIALS AND METHODS

Generation of DC

DC were prepared as described previously [12]. DC were generated by culturing monocytes with 25 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 1000 U/ml IL-4 (R&D Systems, Abingdon, UK) for 5 days at 0.5×10^6 cells/ml in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 15% fetal calf serum. DC were then starved from IL-4 and GM-CSF for 20 h before their stimulation.

Antibodies and other reagents

Monoclonal antibodies (mAb) specific for CD80, CD86, CD83, human leukocyte antigen (HLA)-DR, CC chemokine receptor 7 (CCR7), as well as immunoglobulin G (IgG) control isotypes (PharMingen, San Diego, CA) were used as pure antibodies or as direct conjugates to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Goat anti-mouse IgG $F(ab')_2$ FITC was used as a secondary antibody where necessary. Surface IFNAR1 expression was monitored with 10 μg/ml AA3 mAb (a gift of Laura Runkel, Biogen, Cambridge, MA), and IFNAR2 was monitored with anti-CD-118 mAb (Calbiochem, La Jolla, CA), followed by incubation with 10 μg/ml biotinylated anti-mouse IgG antibody and streptavidin-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) [28]. IFN-α2 (Roferon-A, F. Hoffmann-La Roche Ltd., Basel, CH) and IFN-β (Avonex, Biogen) were generally used at 200 pM, unless specified otherwise. IFN-γ (Peprotech, London, UK) was used at 1000 U/ml; tumor necrosis factor α (TNF-α; Peprotech) was used at 100 ng/ml and IL-1β (Peprotech) at 10 ng/ml; and purified, natural IFN-α was used at 800 U/ml (corresponding to an antiviral activity induced by 200 pM recombinant IFN subtype at 2×10^8 U/mg, such as IFN-α2 or IFN-β) and was provided by the Finnish Red Cross Blood Transfusion Service (Helsinki) [29]. Sheep antiserum raised against human leukocyte IFN [30] was used at a 1:100 dilution. Rabbit polyclonal antisera raised against human IFN-α or IFN-β (PBL Biomedical Laboratories, New Brunswick, NJ) were used at 20 μg/ml. Cells cultured in the presence of these antisera were washed extensively before addition of exogenous IFN-α or IFN-β. LPS from *Escherichia coli* 0111:B4 (Sigma Chemical Co., St. Louis, MO) was used at 1 μg/ml to induce DC maturation. Poly I:C (Sigma Chemical Co.) was used at 50 μg/ml.

Protein analysis

Whole cell extracts were prepared as described previously [11]. Thirty micrograms were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose membranes. The phosphorylation and content of STAT-1 and STAT-2 were detected by immunoblotting (antibodies from Upstate Biotechnology, Lake Placid, NY, to detect the phosphorylation; antibodies from Santa Cruz Biotechnology, CA, to evaluate the content). For IFNAR1 analysis, 60 μg lysates were separated on a 7% SDS-PAGE gel. Blots were incubated with 64G12 mAb (a gift of Pierre Eid, CNRS-UPR, Villejuif, France) [31]. For IFNAR2 analysis, 600 μg total cell extracts were incubated with anti-CD118 mAb (Calbiochem) overnight. The immunoprecipitated material was resolved on a 7% SDS-PAGE gel and blotted with D5 mAb (a gift of L. Runkel). Bands were revealed with an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK) and quantified with the Kodak Image Station 440CF.

RNase protection assay (RPA)

RNA was extracted from DC with RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RPA was performed as described previously [5]. The hCK-5 multiprobe template set (Riboquant, PharMingen) was used to analyze the chemokine expression.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) quantification

Quantitative RT-PCR assays were done as described previously [12]. All quantification data are presented as a ratio to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level. The standard errors (95% confidence limits) were calculated using the Student's *t*-test. Quantification standard curves were obtained using dilutions (4-log range) of the RT-PCR products in 10 μg/ml sonicated salmon sperm DNA. The sequences of the primer pairs used for the quantification of IRF-7 and GAPDH have been described previously [12]. The primer pairs used for IFNAR1 and IFNAR2 were: IFNAR1 forward 5-CGTACAAGCATCTGATGG-3, and reverse 5-GCATTTGAAGTGTTTTCCC-3; IFNAR2 forward 5-TTCCAAACACGAACTACTGT-3, and reverse 5-GGTGCATTTTAAGGGAGACT-3.

Fluorescein-activated cell sorter (FACS) analysis

The maturation and activation state of DC was monitored using antibodies against CD80, CD86, CD83, HLA-DR antigen, and CCR7 as described previously [5]. Surface IFNAR1 and IFNAR2 expression was monitored as described previously [28].

CXCL10 determination

Supernatants from DC cultures were harvested after the indicated treatments and stored at −80°C. CXCL10-specific enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems. The ELISA was conducted according to the manufacturer's instructions. Supernatants from three independent experiments were considered.

RESULTS

Autocrine IFN-β induces a persistent and saturated signaling in mDC

We have recently shown that the stimulation of TLR-3 with poly I:C or of TLR-4 with LPS leads to the rapid transcriptional induction of specific IFN subtypes from myeloid DC [12]. To study the effect of this autocrine type I IFN, we first monitored the activation of STATs in DC stimulated with LPS or poly I:C for various times (**Fig. 1**). Tyrosine phosphorylation of STAT-1 and -2 was detected as early as 3 h following stimulation with TLR-3 and -4 agonists. With the exception of the first hour, the extent and duration of phosphorylation were comparable with that observed in iDC challenged with 0.2 nM IFN-β or IFN-α2.

The addition of exogenous IFN-α2 and -β to 24 h LPS or poly I:C-stimulated cells did not further augment STAT phosphorylation (**Fig. 2A**). To evaluate whether this state was an intrinsic property of mDC, we measured the IFN response in cells that underwent maturation following the combined treatment with TNF-α and IL-1β. In this context, a robust response

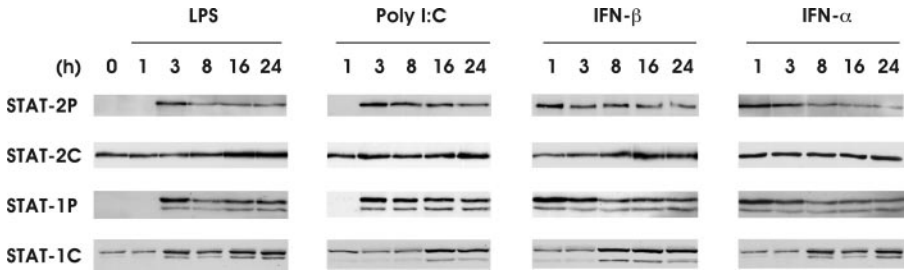

Fig. 1. Activation of STAT-1 and STAT-2 upon LPS- or poly I:C-induced DC maturation. DC were treated with LPS, poly I:C, IFN-β, or IFN-α2 (0.2 nM) for the indicated times. Whole cell lysates (30 μg) were analyzed by immunoblot with the indicated antibodies to evaluate STAT phosphorylation (P) and protein content (C). Note that the anti-STAT-1P antibodies recognize both phosphorylated forms of STAT-1 (p91 and p84). These are representative immunoblot experiments, which were repeated an additional three times with cell extracts from different DC cultures.

to IFN-α2 and IFN-β was observed, as assessed by STAT activation (Fig. 2A, right panels). It is important that these two proinflammatory cytokines do not induce the production of IFN (unpublished observation), suggesting that the persistent IFN signaling in LPS or poly I:C-stimulated DC could be consequent to the autocrine release of type I IFNs.

To evaluate this hypothesis, neutralization experiments were carried out. DC cultures were stimulated for 24 h with LPS in the presence of neutralizing anti-IFN-α/β antibodies (Fig. 2B), anti-IFN-α, or anti-IFN-β antibodies (Fig. 2C). A clear reduction in STAT-1 and -2 phosphorylation was observed in cells stimulated in the presence of anti-IFN-α/β antibodies, suggesting an active signaling role of autocrine type I IFNs (Fig. 2B, lanes 4 and 5). We recently reported that in DC stimulated with LPS, IFN-β is the prominent subtype produced together with a low level of IFN-α1 [12]. Therefore, we investigated which of these two subtypes was responsible for the STAT activation observed in LPS-mDC. The results shown in Figure 2C indicate that the principal inducer of STAT-1 and -2 activation is IFN-β, as its neutralization abolished STAT phosphorylation. In line with this, when IFN-β was neutralized in the culture, STAT activation could be detected in response to IFN-α and -β added exogenously (Fig. 2B, lanes 6 and 7; Fig. 2C, lanes 9 and 10). Conversely, the addition of IFN-α neutralizing antibodies showed no detectable effects. Altogether, these results confirm and extend previous finding [26], showing

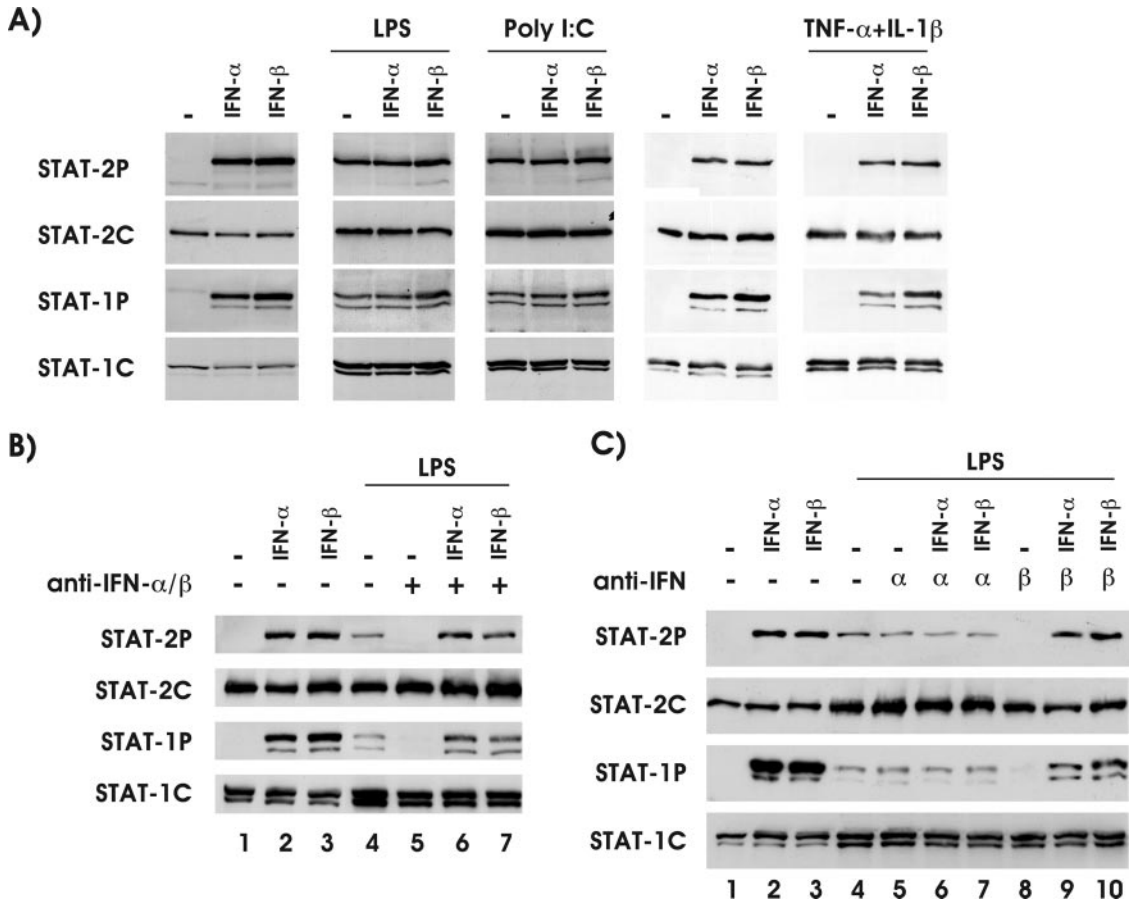

Fig. 2. Autocrine-acting IFN-β saturates the type I IFN response in mDC. (A) DC from one donor were incubated for 24 h with LPS or poly I:C (left panels), whereas cells from a different donor were treated with TNF-α + IL-1β for 24 h (right panels). (B and C) DC were treated 24 h with LPS in the presence or in the absence of neutralizing anti-IFN-α/β (B), of anti-IFN-α, or of anti-IFN-β antibodies (C). Subsequently, the cells were pulsed for 30 min with IFN-α2 of IFN-β, as indicated. Whole cell extracts were analyzed by immunoblot with anti-STAT-1P and STAT-2P antibodies. After stripping, the blots were reprobed with anti-STAT-1 and STAT-2 to evaluate protein content. The results shown are from one of four experiments that yielded similar results.

that in the course of LPS-induced maturation, autocrine IFN-β saturates elements of the IFN signaling pathway.

Washed mDC resume their responsiveness to IFN-β but not IFN-α

Upon maturation, DC acquire the capacity of migrating from infected tissues to lymph nodes through the tightly regulated expression of specific chemokine receptors [32, 33]. Moreover, mDC reach the lymphoid organs when their capacity to release IFN-β is exhausted [2, 12]. Based on these premises, we considered the possibility that mDC resume their responsiveness to IFN once they reach the lymph node. To mimic this scenario, we removed the cytokines secreted during maturation by replacing the culture medium three times (every 8–10 h) within the 24-h period following the LPS treatment. The efficacy of this washing procedure was evaluated by checking the withdrawing of the released IFN-β by ELISA and by controlling the phosphorylation status of STAT-1 and -2 by immunoblots following each wash (**Fig. 3A**). Three washes were required to switch off the long-lasting STAT phosphorylation observed following TLR-4 and -3 triggering (Figs. 1A and 3A). Hereafter, we will refer to these cells as washed mDC. To ensure that the extensive washing procedure did not cause reversion of the mature phenotype, the expression of several maturation markers was analyzed. Withdrawing the cytokines released during the LPS treatment did not affect the expression of CD80, CD86, HLA-DR, and CCR7 (data not shown). Despite a slight reduction of CD83 expression, washed mDC remained clearly positive for this marker (data not shown).

The IFN sensitivity of washed mDC was then assessed by analyzing STAT activation (Fig. 3B). In iDC, a robust and comparable phosphorylation of STAT-1 and -2 was observed following a 30-min pulse with 0.2 nM IFN-α2 or IFN-β, a dose that saturates the transcriptional response in most cell types, including DC (Fig. 3B). In washed mDC, IFN-β strongly activated STAT-1 and -2, whereas IFN-α2 induced an extremely poor STAT activation. At 2 nM dose of IFN-α2, the level of activation was still fourfold lower than in cells treated with 0.2 nM IFN-β. Moreover, the stimulation of washed mDC with a mixture of IFN-α, released from Sendai virus-induced human leukocytes [29], did not cause STAT-1 and STAT-2 activation, indicating that the unresponsive state was not unique to IFN-α2 but was toward all IFN-α subtypes (Fig. 3C). Concordant with the above data, the mRNA levels of two ISGs (IRF-7 and CXCL10, measured by real-time RT-PCR and RPA, respectively) were equally induced in iDC by the two IFN subtypes, whereas an evident differential induction was observed in mature-washed cells (**Fig. 4, A** and **B**). As expected, the expression of IRF-7 and CXCL10 was maximal in DC matured in LPS and could not be enhanced further by exogenously added IFN-α2 or -β. The differential CXCL10 induction by IFN-α and IFN-β in immature, mature, and mature-washed cells was also confirmed at the protein level by measuring the release of this chemokine in culture supernatants (Fig. 4C).

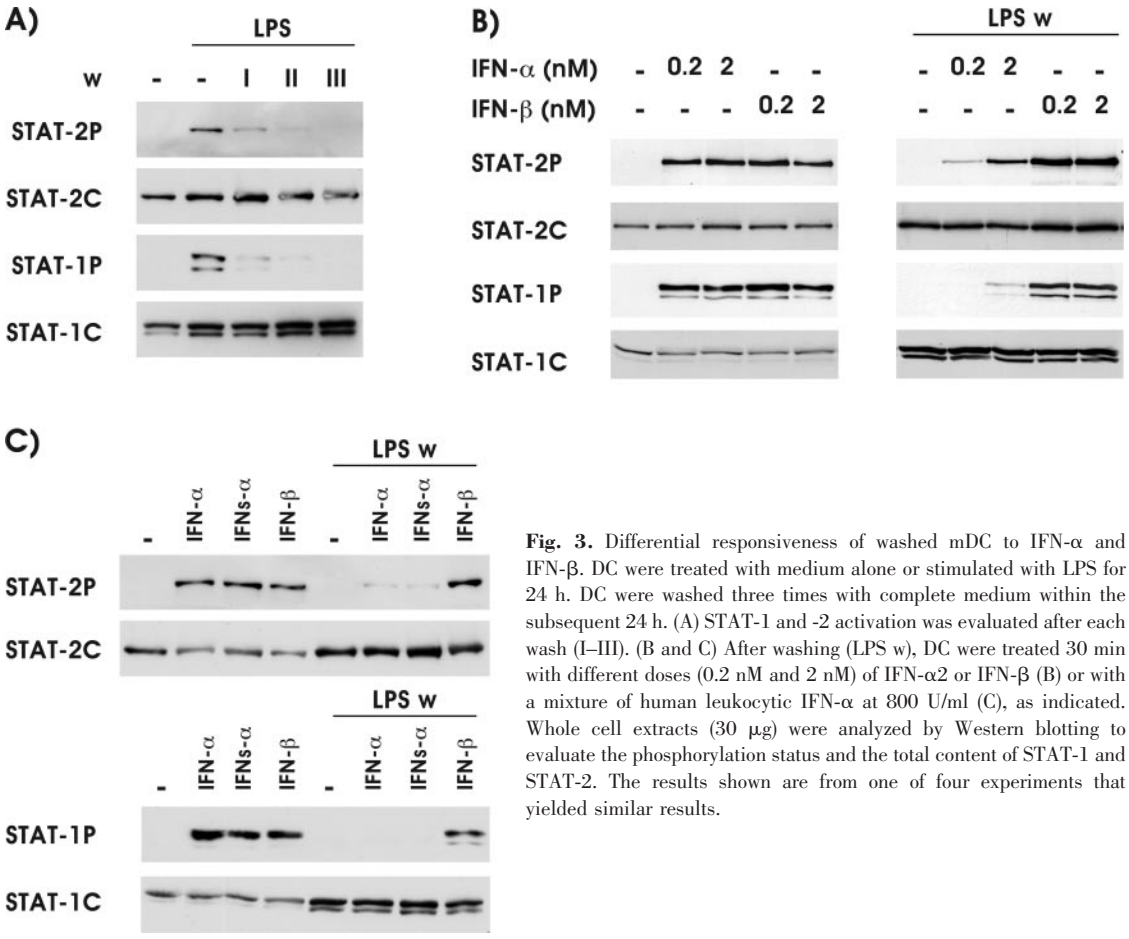

Fig. 3. Differential responsiveness of washed mDC to IFN-α and IFN-β. DC were treated with medium alone or stimulated with LPS for 24 h. DC were washed three times with complete medium within the subsequent 24 h. (A) STAT-1 and -2 activation was evaluated after each wash (I–III). (B and C) After washing (LPS w), DC were treated 30 min with different doses (0.2 nM and 2 nM) of IFN-α2 or IFN-β (B) or with a mixture of human leukocytic IFN-α at 800 U/ml (C), as indicated. Whole cell extracts (30 μg) were analyzed by Western blotting to evaluate the phosphorylation status and the total content of STAT-1 and STAT-2. The results shown are from one of four experiments that yielded similar results.

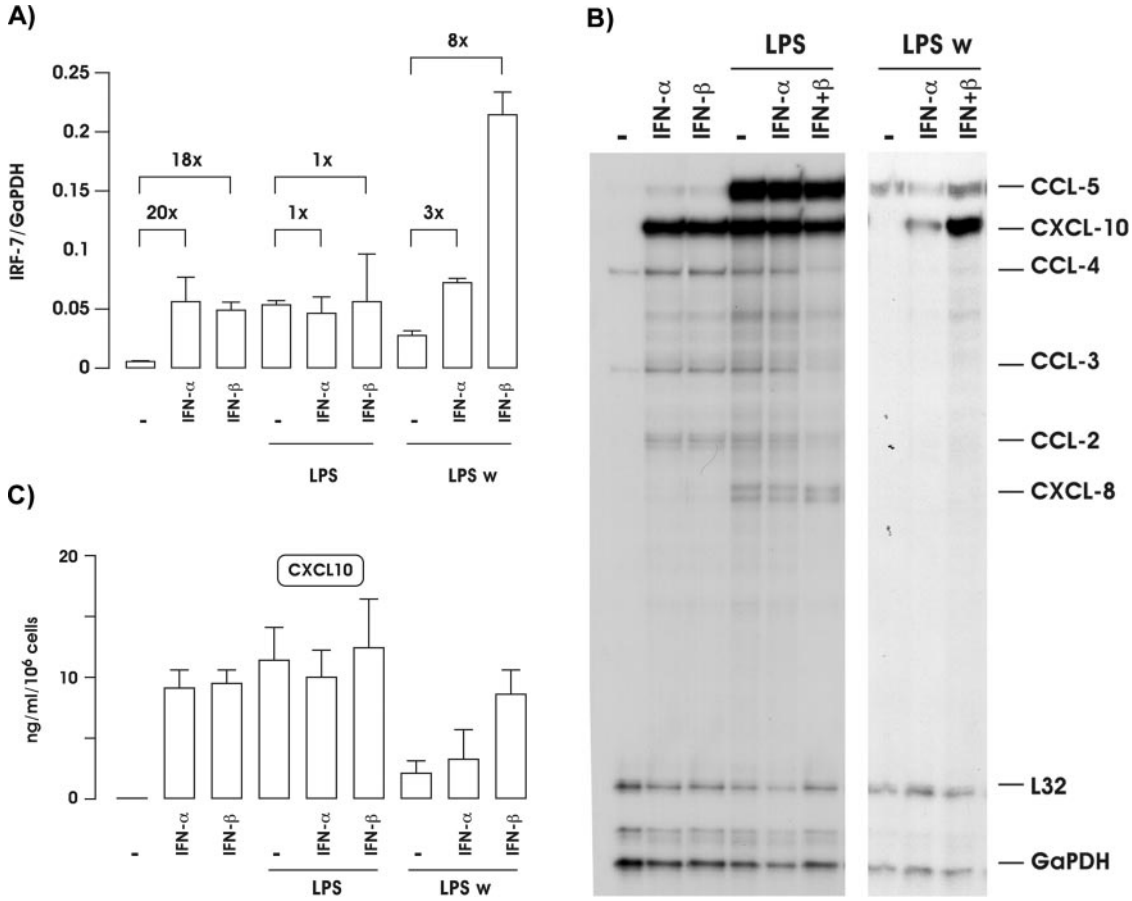

Fig. 4. Differential induction of two ISGs following IFN-α2 and IFN-β treatment in LPS mDC and washed mDC. Total RNA was extracted from control iDC, LPS mDC, and washed mDC (see legend of Fig. 3), which were incubated without or with IFN-α2 or IFN-β for 4 h. (A) IRF-7 mRNA expression was analyzed by real-time RT-PCR. The relative fold-induction values are indicated within each subset. (B) Five micrograms of total RNA was tested by RPA to evaluate CXCL10 expression. These are representative experiments, which were repeated for an additional two times with RNA extracted from different DC cultures. (C) Analysis of CXCL10 production by ELISA. Cell culture supernatants were collected from control iDC, LPS mDC, and washed mDC, which were incubated without or with IFN-α2 or IFN-β for 24 h. The results represent the means + SE of three separate experiments.

Thus, with time and upon clearing of the culture medium, mDC resume their capacity to optimally respond to IFN-β but not to IFN-α2.

Similar to LPS, IFN-β induces desensitization to IFN-α2

The differential responsiveness of washed mDC to the two IFN subtypes may be consequent to the release of IFN-β occurring readily upon TLR-3 and -4 stimulation [12]. To test this hypothesis, iDC were exposed directly to IFN-β for 24 h and then washed for the subsequent 24 h. STAT activation was evaluated in these cells and in washed mDC following a 30-min pulse with IFN-α2, IFN-β, or IFN-γ (**Fig. 5**). It is interesting that as seen for mature-washed cells, also iDC, which were exposed to IFN-β and then washed, became refractory to IFN-α2 but not to IFN-β. Conversely, no differences were observed in IFN-γ-induced STAT-1 activation in all tested conditions (Fig. 5). Two major conclusions can be drawn from these data: first, the desensitization to IFN-α2 of washed mDC is caused primarily by the IFN-β released in the course of maturation; second, molecules widely implicated in the negative regulation of cytokine signaling, such as the suppressor of cytokine signaling (SOCS) or protein inhibitor of activated STAT (PIAS) proteins [24, 27, 34], cannot be invoked, as washed mDC are fully sensitive to IFN-γ and IFN-β. Accordingly, we found that neither SOCS-1 nor SOCS-3 mRNAs, quantitated by real-time RT-PCR, were expressed in washed mDC, indicating that the defective IFN-α2 signaling is not dependent on these molecules (data not shown).

IFNAR1 and IFNAR2 are differentially regulated in LPS-stimulated DC

As described above, the impaired IFN-α2 response of washed mDC is causally related to the IFN-β produced early during the maturation process. To investigate this further, we analyzed the surface levels of the two IFN receptor subunits, IFNAR1 and IFNAR2. In DC exposed for 24 h to LPS or IFN-β, a marked reduction of the surface levels of IFNAR1 and IFNAR2 was observed with respect to the levels present on iDC (**Fig. 6A**). In DC exposed to LPS or IFN-β and subsequently washed, the surface level of IFNAR1 remained low, and the level of IFNAR2 returned to nearly initial values.

As no changes in IFNAR1 nor IFNAR2 mRNA levels were detected in the various experimental conditions, as assessed by

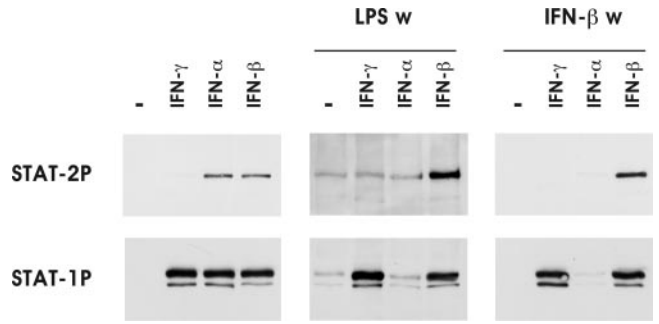

Fig. 5. IFN-β as well as LPS lead to differential IFN-β and IFN-α2 responsiveness of washed mDC. DC were stimulated with LPS or IFN-β for 24 h and washed as described in the legend of Figure 4. The cultures were then pulsed for 30 min with IFN-α2, IFN-β, or IFN-γ. Whole cell extracts (30 μg) were analyzed by immunoblot with anti-STAT-1P and STAT-2P antibodies. Similar results were obtained with extracts from four different DC cultures.

real-time RT-PCR (Fig. 6B), the regulation of the expression of the receptor subunits must occur at a post-transcriptional level. The total IFNAR1 and IFNAR2 protein levels were analyzed in lysates of DC stimulated with LPS or IFN-β for different lengths of time. IFNAR1 was reduced significantly at 2 h and further declined at 6 h and 24 h of either treatment (Fig. 6C, lanes 2–8). A comparable reduction of IFNAR1 was observed when iDC were treated with IFN-α2 (data not shown). It is notable that in washed mDC, IFNAR1 was poorly replenished as compared with iDC (Fig. 6C, upper panel, lanes 9–11). The level of IFNAR2, decreased following a 2-h treatment with IFN-β, was barely detectable at 6 h and started to resume at 24 h (Fig. 6C, lower panel, lanes 1, 3, 5, and 8). In LPS-stimulated cells, IFNAR2 down-modulation was delayed: the level decreased at 6 h and was still low at 24 h (Fig. 6C, lower panel, lanes 1, 2, 4, and 7). This delayed kinetics most likely reflects the time lag needed for the release of autocrine IFN-β [12]. It is remarkable that in mature-washed cells, the level of IFNAR2 was restored to the level present in iDC (Fig. 6C, lower panel, lanes 9–11). This analysis was repeated five times with DC obtained from different donors. Despite some variability among donors in the total level of the receptor subunits, the changes that were observed under the different conditions of stimulation followed a similar pattern.

To test the possibility that the down-modulation of IFNAR1 and IFNAR2 seen at 24 h of LPS treatment was a result of autocrine IFN-β, a neutralization experiment was carried out. DC were left nonstimulated or were stimulated for 24 h with

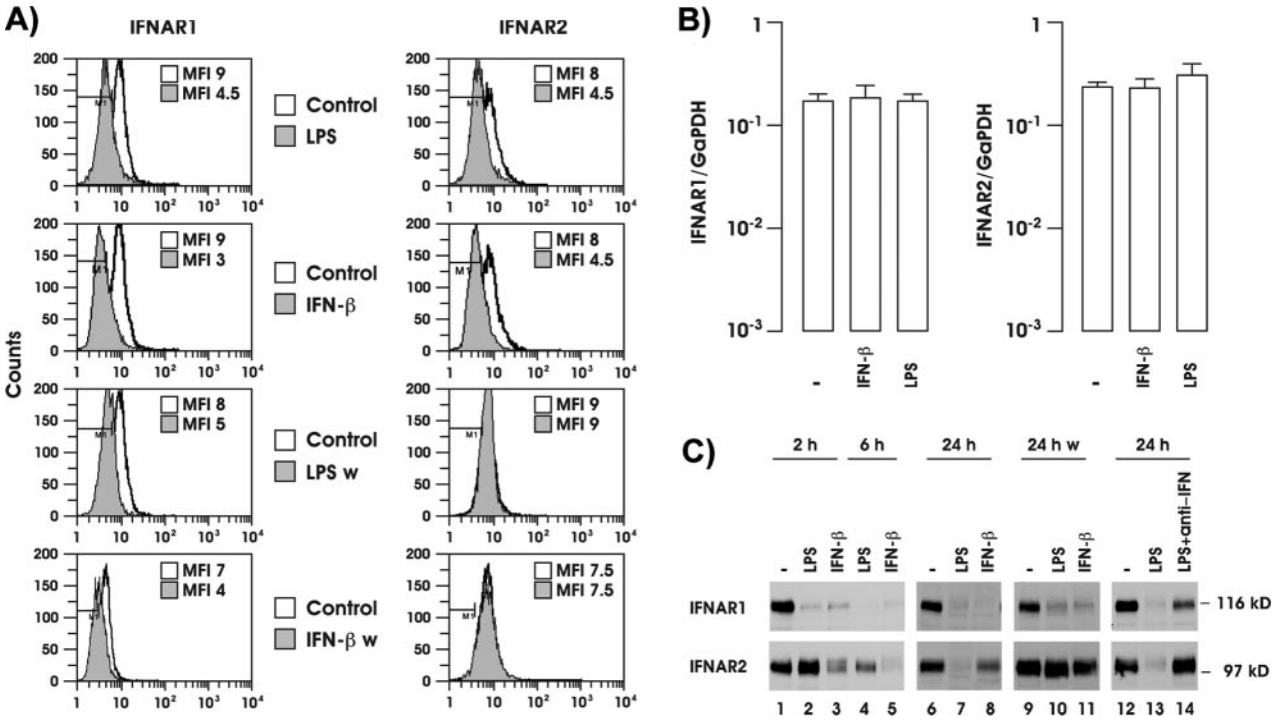

Fig. 6. Levels of IFNAR1 and IFNAR2 in LPS- or IFN-β-treated DC. (A) The surface level of IFNAR1 and IFNAR2 was analyzed by FACS in control DC or in DC stimulated with LPS or IFN-β for 24 h (first and second rows of panels: Control, LPS, IFN-β). In parallel cultures, the FACS analysis was performed following the washing procedure (third and fourth rows of panels: Control, LPS w, IFN-β w). A total of 5000 cells was tested per sample. A representative FACS profile is shown. The mean fluorescence intensity (MFI) values of nontreated and stimulated cells were shown for each panel. Cells stained with the control isotype antibody were contained in the M1 bar. The analysis was repeated four additional times, using DC from a total of five different blood donors. (B) IFNAR1 and IFNAR2 mRNA expression was analyzed by real-time RT-PCR. Total RNA was extracted from control iDC and from DC treated for 24 h with LPS or IFN-β. This is a representative real-time RT-PCR, which was repeated an additional three times with RNA from different DC cultures. (C) Total levels of IFNAR1 and IFNAR2 proteins were analyzed in nontreated iDC and in DC treated with LPS or with IFN-β for 3, 6, or 24 h, as indicated (lanes 1–8); in DC stimulated for 24 h with IFN-β or LPS and then washed as described previously (lanes 9–11); and in DC treated 24 h with LPS in the presence or absence of neutralizing anti-IFN-α/β antibodies (lanes 12–14). For IFNAR1 detection, 60 μg proteins were analyzed by immunoblot. For IFNAR2 detection, 600 μg proteins were immunoprecipitated and analyzed by Western blot. The position of molecular weight (MW) markers is shown on the left side. The apparent MW of IFNAR1 is close to 116 kD, and that of IFNAR2 is close to 96 kD. These are representative immunoblot experiments, which were repeated an additional four times with cell extracts from different DC cultures.

LPS in the presence of neutralizing anti-IFNα/β antibodies or of irrelevant antibodies, and the level of the receptor subunits was monitored. As seen in Figure 6C (lanes 12–14), the presence of anti-IFN-α/β antibodies had a considerable effect on the level of both proteins. However, the effect was more potent toward IFNAR2 than IFNAR1 in all donors analyzed. IFNAR2 was as abundant as in control cells, and IFNAR1 content varied from 30% to 50% of the level in iDC. Thus, neutralizing the autocrine IFN-β with antibodies only partly restored the level of IFNAR1.

Altogether, the above data can be summarized as follows: in LPS-stimulated cells, IFNAR2 is down-modulated, most likely as a consequence of internalization and degradation induced by the released IFN-β. Conversely, IFNAR1 degradation is more rapid and may not be solely accounted by autocrine IFN-β. In mature-washed cells, IFNAR2 has recovered fully at the plasma membrane and inside the cell, whereas IFNAR1 remains low in both compartments and may account for the reduced responsiveness of washed mDC to IFN-α2.

DISCUSSION

The stimulation of specific TLRs leads to the transcriptional induction of IFN genes in plasmacytoid and monocyte-derived DC [12]. We recently reported that in monocyte-derived DC stimulated with LPS or poly I:C, IFN-β is the only type I IFN subtype produced. Indeed, with the exception of the poorly induced IFN-α1, no other IFN-α subtype is produced [12]. Given the early and transient induction kinetics and considering the migratory capacity acquired by stimulated DC, it is conceivable that mDC will exhaust their ability to secrete IFN-β once they reach draining lymph nodes. In this new location, mDC will act as potent initiators of primary T cell responses and will come across a new microenvironment that may contain elevated amounts of type I IFNs released by plasmacytoid DC. Based on these premises, we undertook a detailed analysis of the responsiveness of early mDC and of fully mDC exposed to exogenous IFN. To reproduce this latter situation, we mimicked a change in cytokine environment by replacing the culture medium following the maturation process, obtaining what we operationally define as mature-washed cells. The major conclusions of our analyses follow.

LPS and poly I:C-stimulated DC respond robustly to autocrine-produced IFN-β, as seen by sustained STAT activation. The long-lasting, type I IFN response in mDC seems to be dependent on the continuous presence of IFN-β, as the addition of antibodies that neutralize IFN-β prevented STAT activation. The kinetics of STAT phosphorylation in our experimental model is reminiscent of the prolonged STAT activation described in the highly IFN-sensitive human Daudi B lymphoblastoid cells, where the maintenance of phosphorylated STAT proteins was regulated by the induced secretion of autocrine IFN [35].

As opposed to iDC, which are sensitive to IFN-α2 and IFN-β, mDC are saturated in their response to both IFN subtypes, and this correlates with progressively reduced surface and total levels of the subunits of the IFN receptor. A fall in type I IFN responsiveness and a concomitant reduction of the expression of the receptor subunits were described previously in 24-h LPS-mDC [26].

With time and upon withdrawing of the culture medium, mDC resume full sensitivity to IFN-β but not to IFN-α, owing to poor replenishment of IFNAR1. The observation that IFN-γ signaling was not impaired in washed mDC and in IFN-β-treated DC excludes the involvement of regulatory molecules such as the Tc protein tyrosine phosphatase, SOCS, or PIAS [24, 27, 34]. These results are in apparent contrast with data by Takaoka and colleagues [36], showing a cross-talk between IFN-γ and IFN-α/β signaling through IFNAR1. One possibility is that the contribution of IFNAR1 to IFN-γ signaling is exerted in only some cell types or that the low, resumed level of IFNAR1 in washed mDC is sufficient to ensure proper IFN-γ signaling.

The modulation of the responsiveness of washed mDC to the two type I IFN subtypes appears related to the production of autocrine IFN-β early upon LPS-induced maturation. In fact, iDC matured with LPS in the presence of antibodies neutralizing IFN-β or iDC matured in the presence of TNF-α and IL-1β, when no autocrine IFN is released, are equally responsive to the two IFN subtypes. Thus, an important concept arising from this work is that depending on the initial maturation stimulus, migrating DC will display distinctive abilities to respond to the cytokine environment encountered in lymphoid organs, and this may in turn affect their ultimate function with regards to T cell activation and polarization.

An interesting finding that emerges from monitoring the expression of the receptor is the different turnover of the two subunits. Our data demonstrate that IFNAR2 down-modulation is induced by autocrine IFN-β. The kinetics of IFNAR2 down-modulation is faster in iDC treated with IFN-β than with LPS (Fig. 6C, lanes 1–5) as a result of the time lag necessary for LPS-induced IFN production. Accordingly, IFNAR2 resumes earlier in IFN-β-treated than in LPS-treated cells (Fig. 6C, compare lanes 7 and 8). It is important that surface and intracellular IFNAR2 levels are restored completely in washed mDC or in DC, which underwent LPS-induced maturation in the presence of anti-IFN antibodies.

Different conclusions emerge for IFNAR1: in LPS-stimulated DC, a rapid down-regulation occurs to a level that ensures maintenance of STAT activation. In other cell types, IFNAR1 has been described as a short-lived protein, which is internalized and degraded rapidly via lysosomes upon ligand binding [37, 38]. It is conceivable that modifications in intracellular trafficking occurring in stimulated DC may also affect IFNAR1 turnover. It is interesting that it was recently shown that a transient burst of endocytosis and actin cytoskeleton remodeling takes place at early stages (30 min) of LPS stimulation of human DC [39]. This would then be followed by the progressive loss of endocytic capacity and by an increase in major histocompatibility complex-peptide presentation [40]. It is interesting that mDC lose sensitivity to IL-10, and this was shown to correlate with reduced membrane expression of the IL-10 receptor 1 subunit [41]. Moreover, down-regulation of surface expression of the chemokine receptor CCR5 occurs upon LPS stimulation of human monocyte-derived macrophages. This was described as a rapid and long-lasting effect of LPS, independent of autocrine-chemokine stimulation [42].

The down-regulation of these and possibly other receptors may ensure that mDC become resistant to a specific subset of cytokines and chemokines.

It is important that in washed mDC, the replenishment of IFNAR1 is partial, as opposed to the full replenishment of IFNAR2 (Fig. 6C, lane 10). We propose that the low level of IFNAR1 in washed mDC is insufficient to initiate productive signaling in response to IFN-α2, and in turn, this may account for their desensitization to IFN-α2. Although suggestive, this interpretation stems from the knowledge that IFN-α2 and IFN-β engage the same receptor complex differently [43–45]. It is notable that biophysical studies have shown that IFN-β possesses a 50-fold higher affinity than IFN-α2 toward IFNAR1 and thus, generates a more stable ternary complex at low IFNAR1 concentrations [46]. Recent functional studies in cell lines have directly related binding affinity of the ligand toward IFNAR1 with biological activity [47]. The higher efficiency of IFN-β to engage IFNAR1 could explain the specific IFN-β signaling in cells, where the concentration of IFNAR1 is limiting, such as the Tyk2-minus cells [48] or the washed mDC described in this work.

Gene expression profiling of human cells stimulated with different IFN subtypes suggested quantitative rather than qualitative differences [4, 49, 50]. If a differential signaling between IFN-β and -α exists, it is likely that a specific IFN-β pathway would be unmasked in cells expressing a low level of IFNAR1. Microarray experiments are in progress to clarify this issue.

Why should a mDC respond to IFN-β and not to IFNs-α? Although this remains an open question, it is possible that in the inflamed lymph node, where plasmacytoid DC may release large amounts of type I IFN, the reduced response to these cytokines may restrain their proapoptotic effects [51], modulate their cytotoxic activities [52], or regulate cytokine production following the interaction with activated T lymphocytes [6–8, 53, 54]. It is interesting that the induction of CXCL10 production following IFN-β treatment of washed mDC might be required for the retention of CX chemokine receptor 3^+-activated T cells within T cell areas of the secondary lymph nodes to optimize the T helper 1 immune response [55]. Moreover, this chemokine might also control the recruitment of natural killer cells into the lymphoid tissues, where they can interact with DC to be regulated reciprocally and mutually [56] or where both cell types could control T cell priming [57].

The results described here shed light on a new physiological mechanism that differentially tunes the response to type I IFN subtypes of a specialized leukocytic population such as DC, which regulates multiple aspects of the immune response. This new finding may have an important impact on the therapeutical use of these cytokines in several viral and neoplastic diseases. Moreover, our findings may also give new perspectives in their use as vaccine adjuvant as well as in strategies for the development of DC-based vaccines.

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