

ORIGINAL ARTICLE

Expression profiles of human interferon-alpha and interferon-lambda subtypes are ligand- and cell-dependent

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Recent genome-wide association studies suggest distinct roles for 12 human interferon-alpha (IFN- α) and 3 IFN- λ subtypes that may be elucidated by defining the expression patterns of these sets of genes. To overcome the impediment of high homology among each of the sets, we designed a quantitative real-time PCR assay that incorporates the use of molecular beacon and locked nucleic acid (LNA) probes, and in some instances, LNA oligonucleotide inhibitors. We then measured IFN subtype expression by human peripheral blood mononuclear cells and by purified monocytes, myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), and monocyte-derived macrophages (MDM), and α -dendritic cells (MDDC) in response to poly I:C, lipopolysaccharide (LPS), imiquimod and CpG oligonucleotides. We found that in response to poly I:C and LPS, monocytes, MDM and MDDC express a subtype pattern restricted primarily to IFN- β and IFN- λ 1. In addition, while CpG elicited expression of all type I IFN subtypes by pDC, imiquimod did not. Furthermore, MDM and mDC highly express IFN- λ , and the subtypes of IFN- λ are expressed hierarchically in the order IFN- λ 1 followed by IFN- λ 2, and then IFN- λ 3. These data support a model of coordinated cell- and ligand-specific expression of types I and III IFN. Defining IFN subtype expression profiles in a variety of contexts may elucidate specific roles for IFN subtypes as protective, therapeutic or pathogenic mediators.

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Interferons (IFN) are pleiotropic cytokines that are expressed by a variety of nucleated cells primarily in response to stimulation of a variety of pathogen recognition receptors (PRR). IFN enhance cellular killing of viruses directly by stimulating expression of anti-viral IFN-stimulated genes (ISG), and indirectly through autocrine loops, and by modifying innate and adaptive immune responses. In addition to their antiviral effects,^{1–4} IFN have a role in tumor immunity,^{5,6} autoimmunity,⁷ intracellular bacterial infections^{8,9} and perhaps even atopy.¹⁰

Interferon was first described as an antiviral factor in chick chorioallantoic membrane in 1957.^{11–13} Twenty years later, IFN- α 1 was purified.¹⁴ It became apparent that IFN- α is a family of 13 homologous genes^{15–17} that produces 12 distinct gene products; mature IFN- α 1 and - α 13 are identical. In addition to type I IFN (which also include IFN- β and others), there are three type III IFN: IFN- λ 1, - λ 2 and - λ 3 (also known as interleukin (IL)-29, IL-28A and IL-28B, respectively);^{18,19} IFN- γ is the lone type II IFN.

The human IFN- α subtypes share 70–80% amino acid sequence identity, and about 35% identity with IFN- β .²⁰ Despite their homology and common use of a single heterodimeric receptor, the *in vitro* antiviral and antiproliferative potencies of the subtypes vary,^{17,21–24} as do their effects on T-cell and dendritic cell (DC) function,²⁵ and B-cell proliferation.²⁶ These differences among the subtypes may be a reflection of their variation in affinity for each of the subunits of the IFN alpha receptor (IFNAR), IFNAR1 and IFNAR2,^{17,27,28} or through variable signaling through the complement receptor type 2 (CR2/CD21).²⁹

Multigene families may arise through gene duplication, in which levels of expression are enhanced but mutated sequences are of little or no functional consequence. Alternatively, the duplicated gene may undergo neofunctionalization, in which beneficial effects are gained from mutation, or partial conversion, in which the duplicated gene is partially replaced with the corresponding sequence of another gene; in either case, the progenitor remains to serve its original function.^{30,31} Woelk *et al.*³¹ reported that each of the *IFNA* gene

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families among a group of placental mammals has undergone significant gene duplication and conversion, suggesting functional gains for the IFN- α subtypes.

Genome-wide association studies suggest distinct roles for specific IFN- α and - λ subtypes. For example, the incidence of hepatitis C virus is associated with polymorphisms in *IFNA2*.³² Spontaneous clearance and response to therapy with peg-IFN- α -2b and ribavirin are both associated with polymorphisms in the *IL28B* gene. All but one of these polymorphisms are in the non-coding regions, and appear to be associated with levels of expression of either IFN- λ 2 or IFN- λ 3 (IL-28A or -28B).^{33,34} Additionally, single-nucleotide polymorphisms (SNP) in the *IFNA1* and *IFNA5* genes are associated with systemic lupus erythematosus,⁷ and variations in four *IFNA* genes are associated with atopy.³⁵

Although demonstration of a unique function or role in pathogen defense or disease of any gene product is ultimate proof of its importance, we may draw inferences about IFN subtype function by analyzing patterns of expression in response to pathogens or molecular models of pathogen invasion. Such analyses have been impeded by the high level of identity among the IFN- α subtypes, and between IFN- λ 2 and - λ 3. We overcame these impediments by using two quantitative real-time PCR (qRT-PCR) probe modifications that discriminate single base differences among genes: molecular beacons (MB)^{36,37} and locked nucleic acids (LNA).^{38,39} MB probes are oligonucleotides that contain a hairpin loop that sequesters the fluorophore adjacent to the quencher. On binding to its specific template, the loop opens and distances the quencher from the fluorophore allowing it to emit fluorescence.⁴⁰ LNA probes contain high-affinity nucleic acid analogues that stiffen the probe and raise its T_m , both of which enhance base mismatch discrimination.³⁸ To these primer/probe sets for the IFN- α and IFN- λ subtypes, we added sets for IFN- β and IFN- γ to assemble a comprehensive expression panel of human types I, II and III IFN.

We then used the complete primer/probe array to analyze expression patterns by peripheral blood mononuclear cells (PBMC), three human primary cell types—monocytes, myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), and two cell types derived from primary monocytes—monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC). We stimulated the cells with four model ligands of pathogens that are commonly used for evaluation of pathogen–host interactions—lipopolysaccharide (LPS), imiquimod and CpG DNA oligonucleotides, which are ligands for Toll-like receptor (TLR)4, -7 and -9, respectively, and poly I:C, a double-stranded RNA ligand for TLR3 and the retinoic acid-inducible gene (RIG)-like helicases RIG-I and melanoma differentiation antigen 5 (MDA5). Our data demonstrate that both cell type and ligand contribute a layer of restriction that governs the expression patterns of the subtypes of IFN- α and IFN- λ , which in turn suggest unique roles for IFN subtypes as protective, therapeutic or pathogenic mediators.

RESULTS

Design and validation of primer/probe sets

Primer/probe sets specific for the mature coding sequence of each IFN subtype were designed, synthesized and titrated. As the sequences of the mature products of *IFNA1* and *IFNA13* are identical, we consider them as one gene product, referred to as IFN- α 1. In some instances, the efficiency of the primer/probe sets designed by *Beacon Designer* was suboptimal and required editing such that we shifted the probe or one of the primers in either the 3' or 5' direction. For example, the

efficiency of the initial IFN- α 1 primer/probe set was 1.85 copies per cycle (2.0 is perfect). Since analysis with *mfold*^{41,42} revealed hairpin loops within the forward primer-binding site, the forward primer was shifted 41 bases in the 3' direction (Supplementary Figure 1). This shift enhanced efficiency to 1.99, which corresponds to an increase from 4.9×10^{10} to 9.0×10^{11} copies after 40 PCR cycles, or an 18-fold increase in sensitivity.

Specificity was determined by testing each primer/probe set for amplification of 1.0pM complementary DNA (cDNA) of IFN- β , IFN- γ , and each IFN- α and IFN- λ subtype. To enhance specificity beyond 512-fold (nine PCR cycles), we adjusted concentrations and/or edited sequences of the primer/probe sets. Amplification and detection of IFN- α 16 cDNA by the primer/probe set for IFN- α 4 was overcome with an LNA competitor against the nonspecific IFN- α 16 sequence.⁴³ Amplified PCR products from stimulated primary cells were sequenced to demonstrate that the amplicon aligned with, and included bases unique to, the appropriate subtype template (not shown). The specificity of each primer/probe set for its specific template vs an equimolar amount of each nonspecific template included in the array is shown in Figure 1.

All of the human IFN- α subtypes have allelic variants.¹⁷ *IFNA4* has two allelic variants that are equally distributed in the population, and are expressed as amino acid substitutions A51E and T114V for IFN- α 4a and - α 4b, respectively.⁴⁴ As these non-synonymous substitutions are either immediately proximal to (A51E), or within (T114V) an α -helix, one or both may affect the contact points to IFNAR2 or IFNAR1, respectively,⁴⁵ and thus may affect their relative biological activity. In the event that this subtype emerges as relevant to a particular context, we designed MB primer/probe sets which, combined with an LNA oligonucleotide competitor, differentiate between IFN- α 4a and IFN- α 4b (Supplementary Figure 2).

Supplementary Figure 3 shows the layout of the 384-well PCR plate that measures two housekeeping genes (HKG) and all IFN from 16 samples, and includes four-point standards and negative controls for each of the IFN. The sequences of the primer/probe sets, LNA competitors, their positions relative to the first codon of the mature gene product, and their concentrations used in this assay are shown in Supplementary Table 1. The efficiency and sensitivity for each primer/probe set are shown in Supplementary Table 2. The precision (reproducibility) of each primer/probe set was tested on six plates, each of which included four replicates of each four-point standard curve (Supplementary Table 3).

Human PBMC exhibit ligand-specific IFN expression patterns

To determine patterns of expression of types I, II and III IFN, we stimulated PBMC from six donors with poly I:C, LPS, imiquimod and CpG oligonucleotides, and harvested cells and supernatants at 1, 4, 8, 16 and 24 h. Data are shown as 'radar' plots generated using Microsoft Excel (Microsoft, Redmond, WA, USA), with the IFN- α subtypes arranged clockwise according to a phylogenetic tree of their protein sequence (Supplementary Figure 4, Geneious Pro, Version 5.3.4, Biomatters Ltd, Auckland, New Zealand). Geometric means of peak responses from six donors (Figure 2a) demonstrate that the response to LPS is essentially limited to IFN- β and IFN- λ 1, and that all IFN species are expressed in response to CpG oligonucleotide. Between these two extremes, poly I:C and imiquimod elicit expression of a similar subset of IFN- α subtypes, but imiquimod does not elicit comparable expression of IFN- λ .

Figure 2a also demonstrates the advantage of considering gene expression in terms of copy number (per μ g RNA) rather than normalizing to expression of a HKG (Δ quantification cycle (Cq)).

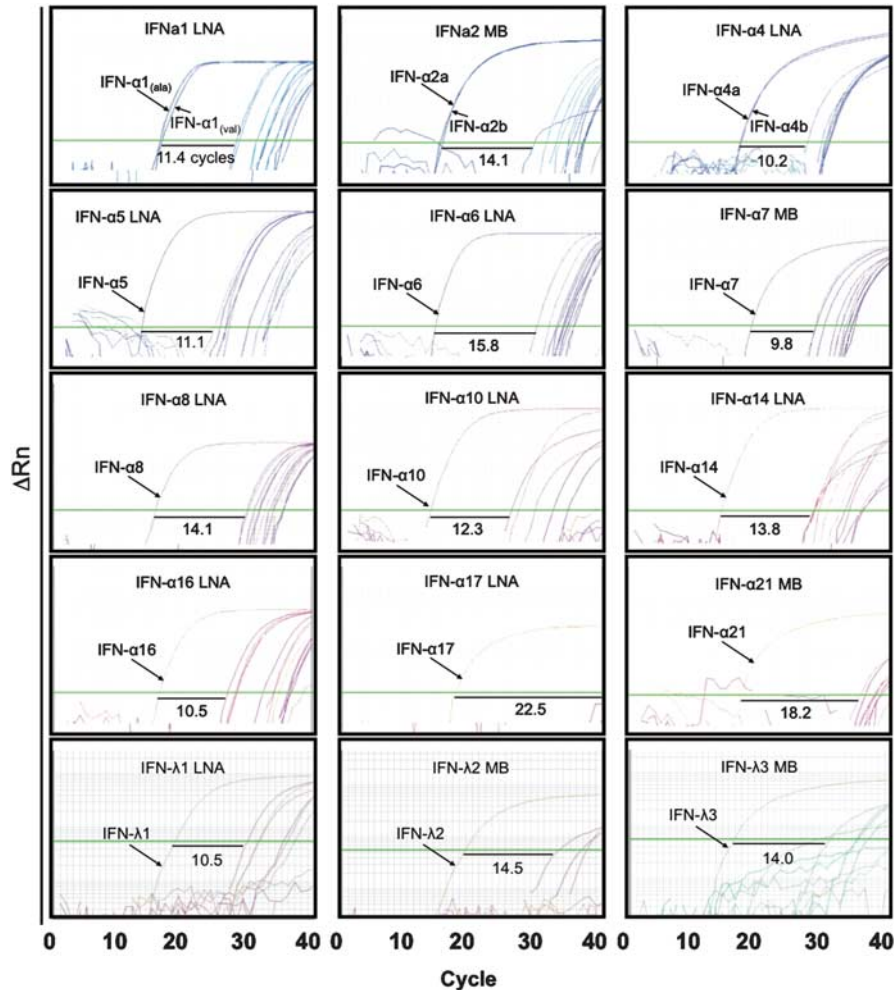


Figure 1 IFN- α and IFN- λ primer probe sets are highly specific. For each IFN- α and IFN- λ subtype, 1.0 pM of cDNA was amplified with its specific primer/probe set, and each nonspecific primer/probe set. The difference in cycle numbers between the target cDNA template and best amplified nonspecific template for each primer/probe set demonstrates a minimum of ~ 1000 -fold specificity.

For example, the suggestion of poor IFN- $\lambda 2$ and $\lambda 3$ expression in response to poly I:C or CpG (left plot) is a reflection of the relatively low efficiency of their respective primer/probe sets (1.85 and 1.94, respectively). Standard curves used to calculate copy number take PCR reaction efficiency into account, and demonstrate that these two IFN are expressed at relatively high levels (right plot).

We then asked whether the gene expression patterns correlate with levels of IFN proteins in the cell supernatants. Supplementary Figure 5 shows that among the six donors tested, very little IFN- α and IFN- λ protein were detected before 16 h. IFN- β was detected across multiple time points, but without clear patterns for all TLR ligands except CpG oligonucleotides. In general, higher levels of IFN protein were detected from the PBMC that were freshly stimulated vs frozen.

To correlate the expression of IFN- α protein and its genes, we plotted the sum of the copy numbers of all IFN- α subtypes against protein levels measured by enzyme-linked immunosorbent assay (ELISA) at 8, 16 and 24 h. For the poly I:C and CpG oligonucleotide-stimulated cells, gene and protein expression correlated qualitatively (Figure 2b), and at the 8 h time point, quantitatively (Figure 2c). Comparison of gene and protein expression of IFN- β in response to CpG oligonucleotide (Figure 2d) and IFN- λ in response to poly I:C (Figure 2e) also suggest time-dependent correlations.

Taken together, these data suggest that gene and protein expression correlate at later time points that reflect the time necessary for translation and protein secretion. In addition, failure to detect protein at 4–8 h may be due to receptor-mediated consumption by the cultured cells, and/or the limited sensitivity of protein detection assays as compared with PCR gene expression detection methods. To test whether the failure to detect IFN protein at earlier time points is due to its consumption, we measured expression of two ISG, *IFN response factor-7* (*IRF7*) and *myxovirus resistance-1* (*MX1*). Figure 3 demonstrates that for CpG and imiquimod, type I IFN gene expression correlates with ISG expression, suggesting that secreted type I IFN is unavailable for detection by ELISA because it is consumed by the cultured cells.

Human DC and macrophages exhibit ligand-specific IFN induction patterns

To test homogeneous subsets of IFN-expressing cells, we purified in sequence pDC, mDC and monocytes from a preparation of elutriated monocytes, and verified purity of each by flow cytometry. Purity of the three isolates of mDC (that is, mDC1 subset, CD1c⁺, CD303⁻) and of the four isolates of pDC reported in Figures 4, 5 and Supplementary Figure 6 each ranged between 95 and 98%. We

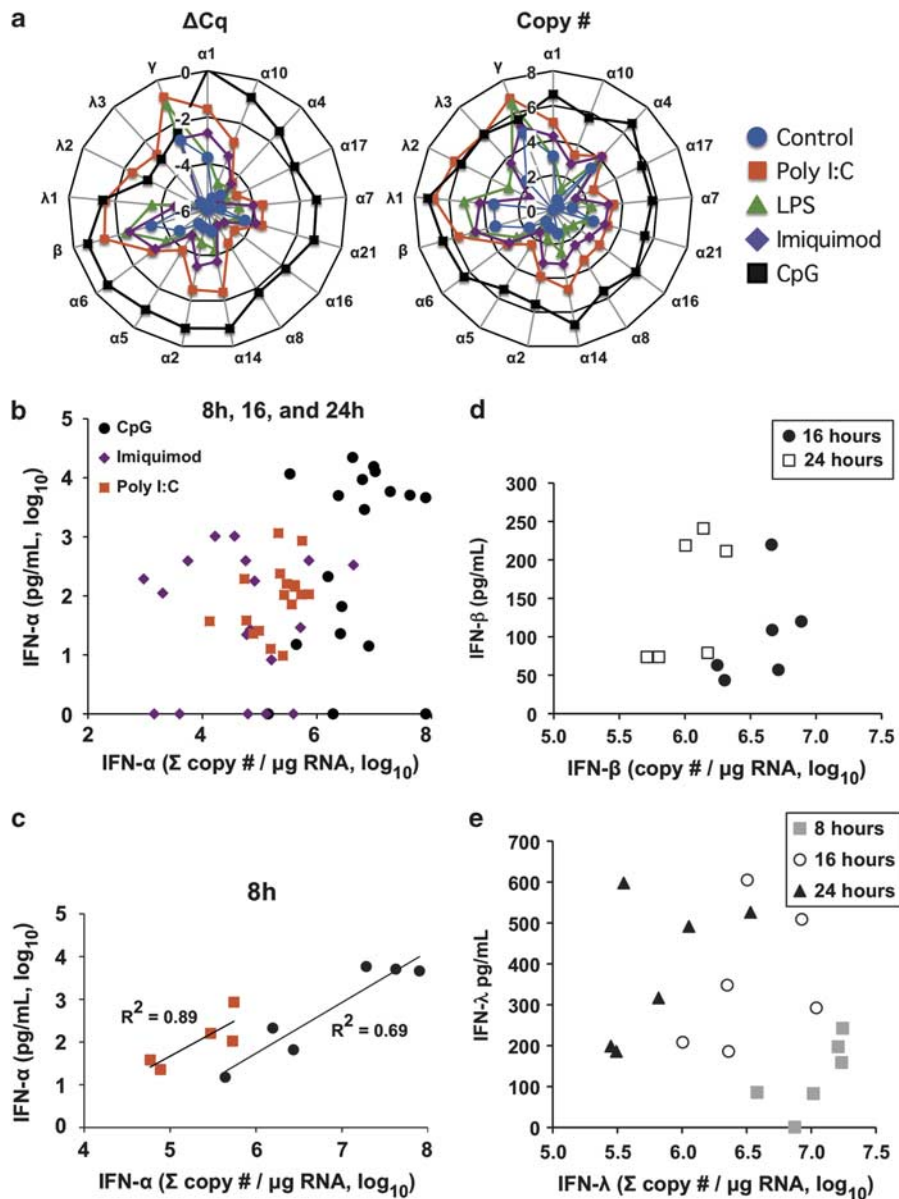


Figure 2 The expression pattern of IFN mRNA and protein differs with each TLR ligand. (a) PBMC were stimulated with each TLR ligand and the cells were harvested for qRT-PCR analysis. The geometric means of the peak responses to poly I:C (8 h), LPS (4 h), imiquimod and CpG and unstimulated control (16 h each) from six donors are shown in \log_{10} scale as a function of expression of the *HKG UBC* (ΔC_q , left), or as copy number μg^{-1} RNA (right). IFN- α subtypes are ordered according to the phylogenetic plot of amino acid sequence similarity shown in Supplementary Figure 4. (b–e) IFN- α , - β and - λ proteins were measured by ELISA, and detectable protein levels were plotted as a function of gene expression. (b) The expression of each IFN- α subtype in copy number μg^{-1} RNA was added, and the sum was plotted against protein expression. (c) Correlation of transcript (sum of all subtypes) and protein levels of IFN- α at 8 h post-stimulation with poly I:C and CpG oligonucleotides. (d) Levels of IFN- β transcript and protein expressed by PBMC at 16 and 24 h in response to CpG oligonucleotides correlate. (e) Levels of IFN- λ (sum of IFN- λ 1, - λ 2 and λ 3) transcript and protein expressed by PBMC at 8, 16 and 24 h in response to poly I:C correlate.

differentiated a portion of the monocytes into MDM with macrophage colony-stimulating factor, and MDCC with granulocyte-macrophage colony-stimulating factor and IL-4, and treated the cells with the TLR ligands for 4 h.

Comparison of monocytes, MDCC and MDM from the same donor (Figure 4a) as either a function of *HKG* expression (ΔC_q) or copy number μg^{-1} RNA (top and bottom, respectively) demonstrates that the IFN response by each of these myeloid cell types was primarily to poly I:C and LPS, and was dominated by IFN- β and IFN- λ 1, but also included low levels of expression IFN- α . In addition to these,

however, the poly I:C-stimulated mDC expressed IFN- α 10, and among two of the three donors tested, IFN- α 21 (Figure 4a and Supplementary Figure 6). Figure 4b shows the geometric mean of the levels of expression by monocytes, MDM and MDCC (there were too few donors for meaningful calculation of the geometric means for mDC) from the subset of donors for whom the quantity of purified RNA allowed for analysis of copy number μg^{-1} RNA.

Statistical analysis of the expression levels (ΔC_q , Figure 4c) demonstrates that monocytes, MDCC and MDM consistently respond to poly I:C and LPS and that those responses are limited to IFN- β ,

IFN- λ 1 and IFN- α 1. MDM, however, express TLR9,⁴⁶ and in response to CpG oligonucleotides express additional IFN- α 10 and - α 14 subtypes (Figure 4d).

In contrast to the myeloid cells, Figure 5 demonstrates that as previously shown,^{47,48} pDC highly express all type I IFN, although IFN- α 6 and IFN- α 8 may be expressed at moderately lower levels. Despite descriptions of identical MyD88 signaling pathways through TLR7 and TLR9, the TLR7 ligand imiquimod, unlike CpG, did not elicit expression of all type I IFN; in the case of the donor shown in Figure 5a, IFN- α 7 was not expressed, while two other donors 'dropped' additional subtypes (Figure 5b).

Supernatants were collected from the donors shown in Figures 4 and 5 for measurement of IFN- α , IFN- β and IFN- λ by ELISA. Supplementary Figure 7 shows data from five MDDC sets (control, LPS and poly I:C) demonstrating that IFN- β and IFN- λ transcript and protein levels correlated. The extent of correlation, however, could not be determined because the samples were unavailable to re-assay. For MDM and mDC, there was very little protein detected in the supernatants, and no correlation between protein and transcript levels. For pDC, protein levels of IFN- α and IFN- β were either below limits of

detection (control) or above the limits of quantification (CpG or imiquimod), and samples were unavailable for re-assay.

DISCUSSION

Differential gene expression of human IFN- α subtypes was first demonstrated over 25 years ago⁴⁹ and was later confirmed at the protein level.⁵⁰ More recently, the SYBR green approach⁴⁷ and nested multiplex PCR followed by SNP-PCR⁴⁸ were used to demonstrate promiscuous expression of IFN- α subtypes by pDC in response to influenza virus. Here, we used structurally (MB) or chemically (LNA) modified probes, and when necessary, LNA inhibitors, to develop and validate a novel assay that is amenable to high-throughput, quantitative analysis for expression of human IFN genes. In consideration of the IFN as a family of immunomodulators, we tested for expression of all the subtypes of IFN- α and IFN- λ , and also for IFN- β , and IFN- γ . We then used PBMC followed by three primary and two laboratory-derived cell types and four TLR ligands to demonstrate that both cell type and ligand are critical in determining IFN expression profiles.

To compensate for differences in efficiency among the primer/probe sets, we included a four-point standard curve so that expression of each IFN can be described as a function of a HKG (Δ Cq), or copy number μg^{-1} RNA. We believe that copy number is a better index of gene expression because HKG expression may vary according to stimulation and cell type,⁵¹ requiring, as we did here, careful choice of HKG for each experimental design. More importantly, standard curves naturally take into account primer/probe efficiency, whereby very small differences amplified over multiple PCR cycles falsely imply differences in gene expression (Figure 2).

Mouse models support the concept that IFN subtypes, rather than serving as amplifiers, qualitatively modify the 'antiviral state'.^{52,53} Baig and Fish⁵⁴ demonstrated that murine IFN expression profiles differed according to the viral stimulus, and also according to whether the IFN were expressed by lung fibroblast cell lines or tissue-stimulated *in vivo*. Similarly, Li and Sherry⁵⁵ reported that murine cardiac myocytes and fibroblasts express different IFN- α subtypes in response to reovirus, and also demonstrated that viral sensitivity varies according to IFN- α subtype.

To our knowledge, this is the first comprehensive report to demonstrate that both the stimulus and the cell type determine the consequent *human* IFN expression patterns. We confirm previous reports that pDC express high levels of IFN- β , IFN- λ and all IFN- α subtypes,^{47,48} but comparatively lower levels of IFN- α 6.⁴⁸ By contrast, the four myeloid lineages expressed high levels of IFN- β and IFN- λ 1, and when expressed, low or intermediate levels of IFN- α 1. In addition, MDDC and mDC frequently expressed IFN- λ 2 and IFN- λ 3, and mDC expressed additional IFN- α subtypes.

We tested IFN expression patterns in response to stimulation through TLR3 (and RIG-I and MDA5), TLR4, TLR7 and TLR9 with their respective ligands poly I:C, LPS, imiquimod and CpG oligonucleotides in a heterogeneous population of human PBMC and in accordance with Puig *et al.*,⁵⁶ demonstrated differences in IFN

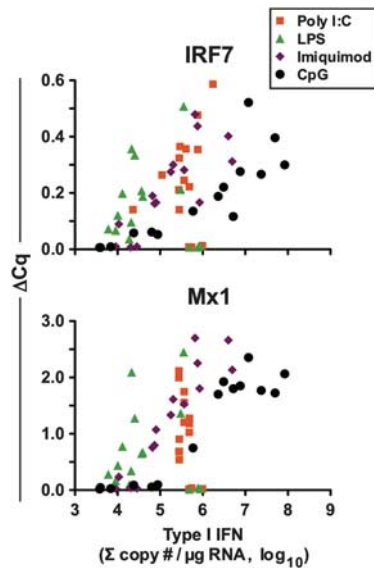
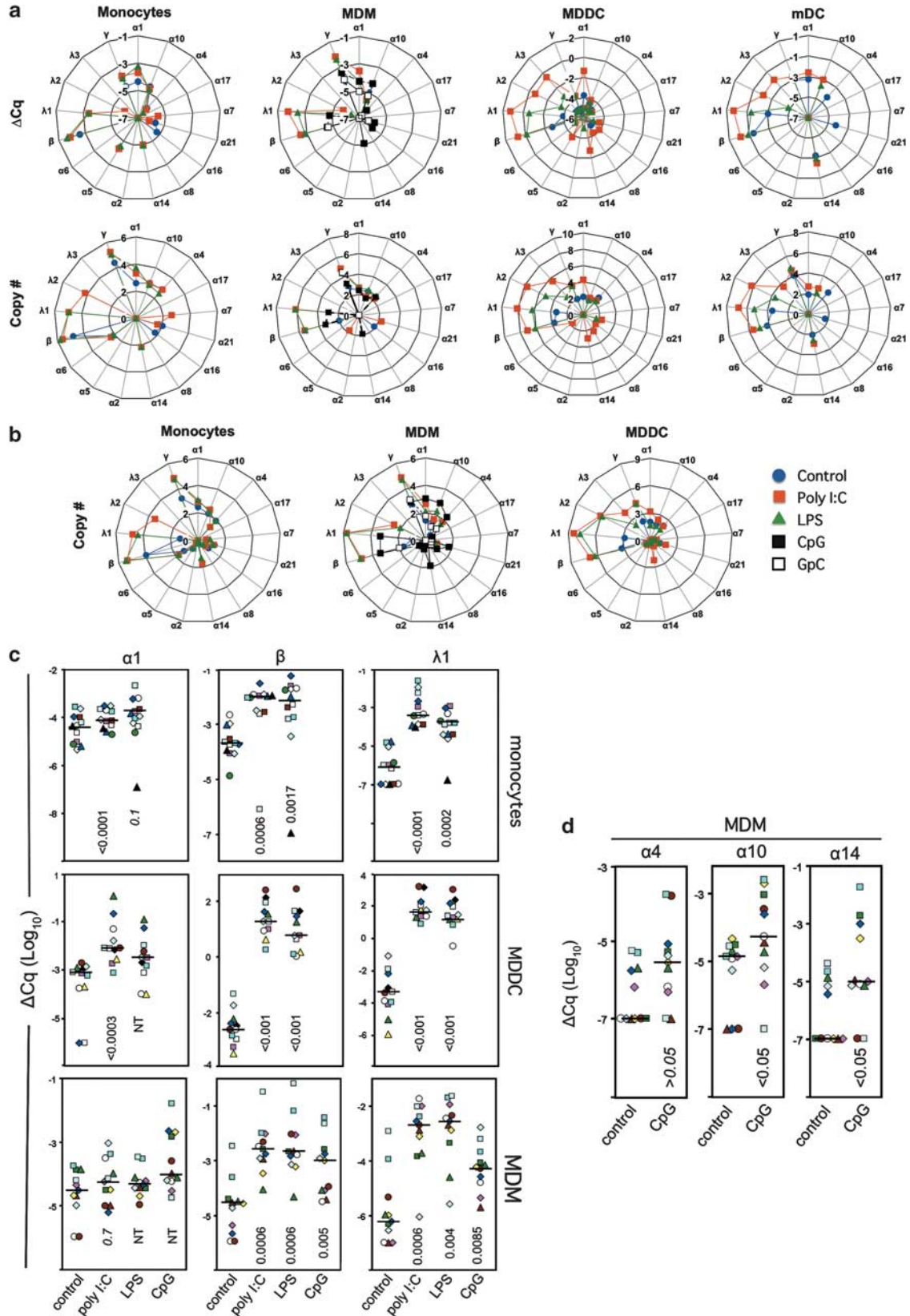


Figure 3 PBMC expression of type I IFN and ISG correlates after stimulation through TLR7 and TLR9. The expression of each type I IFN subtype in copy number μg^{-1} RNA was added and the sum was plotted on the X axis against expression (Δ Cq) of the ISG *IRF7* (Y axis, top) and *MX1* (bottom). Type I IFN expression correlates with either of the ISG after stimulation with imiquimod (diamond) or CpG (circle), but not LPS or poly I:C (triangle and square, respectively). For IFN vs *IRF7* after stimulation with imiquimod: $R^2=0.87$; after stimulation with CpG: $R^2=0.87$. For IFN vs *MX1* after stimulation with imiquimod: $R^2=0.81$; after stimulation with CpG: $R^2=0.83$. Data shown are from PBMC from the three donors in which the cells were not frozen.

Figure 4 Myeloid cells primarily express a limited profile of IFN in response to LPS and poly I:C. (a) Monocytes and mDC were harvested from the same donor, and aliquots of the monocytes were differentiated into MDDC and MDM. Each cell type was stimulated for 4 h with LPS, poly I:C, and in the case of the MDM, CpG oligonucleotides. IFN expression is shown in \log_{10} scale as a function of expression of HKG *SDHA* for mDC and MDDC, and *UBC* for monocytes and MDM (Δ Cq, top), or copy number μg^{-1} RNA (bottom) in \log_{10} scale. Note the difference in scale of each graph. (b) Compiled expression profiles shown as the geometric mean of copy number μg^{-1} RNA for monocytes (eight donors), MDDC and MDM (five donors each). (c) Expression of IFN- α 1, - β and λ 1 (Δ Cq) among a set of donors, each of which is represented by a different symbol, and tested for statistical significance (vs control); *P*-values are shown at bottom of each graph after Bonferroni's correction and are italicized if tested but not statistically significant; NT=not tested. (d) MDM expresses IFN- α 10 and - α 14 in response to CpG; independent statistical analysis of the expression of three IFN- α subtypes that the radial plots suggest are expressed by MDM.

expression patterns that were reflected by differences in protein levels in supernatants, and more significantly, by differences in expression of two ISG, *IRF7* and *MX1*.

We also tested responses to these TLR ligands among purified monocytes, mDC and pDC, and MDDC and MDM, and found that LPS and poly I:C triggered qualitatively identical patterns of IFN



expression—primarily IFN- β , IFN- λ 1 and IFN- α 1 by the myeloid cells. pDC were recently shown by qRT-PCR and immunofluorescence to express MDA5,^{57,58} and in the present study expressed in response to poly I:C a pattern of IFN similar to that of the myeloid cells.

Primarily, type I IFN are expressed in response to PRR stimulation as a consequence of activation of IRF3 and/or IRF7. IRF3 expression is constitutive in all cell types and expression is not enhanced by viral infection^{59,60} (Hillyer and Rabin, unpublished observations), while IRF7 is constitutively expressed in pDC, but otherwise is a classic ISG. In a series of elegant experiments, Genin *et al.*^{20,61} demonstrated that IFN- β and the IFN- α subtypes respond differently to activated IRF3 and IRF7. Alone, activation of IRF3 primarily induces expression of IFN- β and - α 1. On co-activation of IRF3 and IRF7, additional subtypes are expressed. On high levels of co-activation, or when activation of IRF3 exceeds IRF7, expression of these additional subtypes is attenuated. Similar to IFN- β , IFN- λ 1 is expressed in response to activation of either IRF3 or IRF7, and like most *IFNA* genes, IFN- λ 2 and/or IFN- λ 3 are expressed mainly in response to activated IRF7.⁶² This broad specificity of the types I and III IFN genes for IRF3 and/or IRF7 reflects the restricted IFN expression patterns observed in response to poly I:C and LPS, compared with the promiscuous patterns expressed by pDC, which constitutively express high levels of IRF7,⁶³ in response to imiquimod and CpG.

IRF7 specificity alone cannot, however, explain such variations among the IFN expression patterns such as the differences in IFN- α subtype profiles stimulated by CpG vs imiquimod, or the lack of IFN- λ 3 expression in response to either. Nor can IRF3 specificity alone explain the variable expression patterns of the IFN- α subtypes by mDC to poly I:C. These finer differences in expression patterns among cell types may be explained, for example, by differences in expression among positive regulators of type I IFN transcription such as IRF1, IRF3, IRF5, IRF7 and IRF8.^{64,65} In addition, both IRF5⁶⁶ and IRF7⁶⁷ have splice variants, of which only the former has been shown to differentially regulate IFN- α subtype expression. Furthermore, acetylation of IRFs reduce DNA-binding, and depending on the pattern, ubiquitination either enhances their activity or targets them for proteasome degradation.⁶⁸

Our report raises at least four questions in the consideration of IFN responses to infection. First, is it possible that restricted subtype expression, rather than type I IFN *per se*, fails to ameliorate an infectious disease or for that matter, a type of cancer? For example, the pattern of IFN expressed by MDM may help explain the apparent paradox in which type I IFN are both necessary for murine macrophages to respond to *Mycobacterium tuberculosis*,⁶⁹ but also worsens listeriosis and tuberculosis, respectively.^{70–72} Second, because myeloid cells are relatively abundant, moderately express type I IFN, and highly express type III IFN, should they be included with the relatively rare pDC when considering the global IFN response? Third, genetic studies of hepatitis C disease clearly point to the importance of IFN- λ 3, which is hierarchically expressed after IFN- λ 2 and IFN- λ 1. Therefore, what is the added value of IFN- λ 3: does it have unique properties unto itself, or does it modify the function of IFN- λ 2? By extension, does IFN- λ 2 alone or together with IFN- λ 3 modify the response to IFN- λ 1? Alternatively, it is possible that natural infection triggers expression patterns of IFN- λ 2 and/or IFN- λ 3 that are qualitatively different than those induced by simplified ligands such as poly I:C or LPS.

The fourth question is whether co-expression of IFN- α with IFN- λ may represent two limbs of a coordinate response by multiple cell types, in which IFN- λ amplifies, modifies and prolongs the effects of type I IFN,⁷³ and because of restricted expression of type III IFN receptors,⁷⁴ selectively affects the non-hematopoietic cells that are

usually the targets of viral infection. It may be prudent, therefore, to consider the subtypes of IFN- α and IFN- λ not as variations of a pair of cytokines, but as multiple components of an integrated system in which many are functionally specialized and subtly unique.

METHODS

IFN template cDNA

Recombinant cDNA fragments corresponding to each *IFNA* gene were cloned into vectors and purified by PBL (Piscataway, NJ, USA). IFN- λ 1, - λ 2 and - λ 3 cDNA cloned into the pEF-SPFL vector¹⁹ were the kind gift of Sergio Kotenko. IFN- λ plasmids were grown in LB medium (Luria-Bertani medium; Invitrogen, Carlsbad, CA, USA) supplemented with 100 μ g ml⁻¹ ampicillin, isolated with a Miniprep kit (Qiagen, Valencia, CA, USA) and linearized with *Bam*HI. The cDNA for IFN- β and IFN- γ coding sequences flanked with *Eco*RI sites were synthesized and cloned into the vector pUC57 by Genscript (Piscataway, NJ, USA), and linearized with *Sca*I.

qRT-PCR assay to measure expression of subtypes of IFN- α and IFN- λ

Primer/probe sequences for measuring expression of *IFNA*, *IFNB*, *IFNG*, *IL29* (IFN- λ 1) and *IL28* (IFN- λ 2 and - λ 3) were designed using Beacon Designer software (PREMIER Biosoft, Palo Alto, CA, USA) and queried by the NCBI-BLAST algorithm⁷⁵ to verify specificity of candidate sequences against all human genes. Only coding regions for the mature proteins were entered for primer/probe searches. MB⁴⁰ and LNA³⁸ primer/probe sets were designed for *IFNA* and *IL28* and *IL29* genes; linear primer/probe sets were designed for *IFNB* and *IFNG*. All probes but one were conjugated to 6-carboxyfluorescein (FAM) and either tetramethylrhodamine (TAMRA) or Black Hole quencher (BHQ) at the 5' and 3' ends, respectively. The fluorophore and quencher for *IFNG* are tetrachloro-6-carboxy-fluorescein (TET) and 4-(4-dimethylaminophenyl) diazenylbenzoic acid (Dabcyl), respectively. Primers and MB and linear probes were synthesized by the Facility for Biotechnology Resources at the Center for Biologics Evaluation and Research (FBR, Bethesda, MD, USA). LNA probes and competitors were synthesized either by Eurogentec (San Diego, CA, USA) or Sigma-Proligo (St Louis, MO, USA); all probes were purified by high-performance liquid chromatography. Primer/probe sets for the HKG and PCR master mix (Universal Fast Mastermix) were purchased from Applied Biosystems (Foster City, CA, USA).

Forward and reverse primer concentrations (100–500 nM) were optimized for each set by titrating with fixed probe and template concentrations. Probe concentrations (31.25–250 nM) were then titrated with fixed primer and template concentrations. Optimal thermal cycler reaction conditions for the entire set are: 50.0 °C \times 2 min initiation, initial denaturation at 95.0 °C \times 3 min followed by 40 cycles of 95.0 °C \times 15 s and 59.0 °C \times 1 min.

The sensitivity of each primer/probe set was determined by identifying the lowest concentration of triplicates of serial 10-fold dilutions that consistently amplified. To determine PCR efficiency, the IFN cDNA log₁₀ concentrations of template were plotted against their C_q, and the slope of the best-fit line was entered into the equation: Efficiency = 10^(-1/slope).⁷⁶ Specificity of each IFN- α and IFN- λ subtype's reagent set was determined by reacting it individually with 1.0 pM of its corresponding IFN cDNA (target) or all other non-target IFN cDNA on the same PCR plate.

For primer/probe sets that failed to provide \geq 9 cycles of separation (that is, 2⁹ or 512-fold specificity) between the target and non-target templates, the primer and/or probe sequences were edited to avoid predicted secondary structures in the cDNA target sequences, as determined by the software *mfold* (version 3.2).^{41,42} If specificity was insufficient after editing the sequences, amplification of nonspecific sequences was blocked with LNA oligonucleotide competitors.⁴³

Measurement of IFN-subtype expression profiles

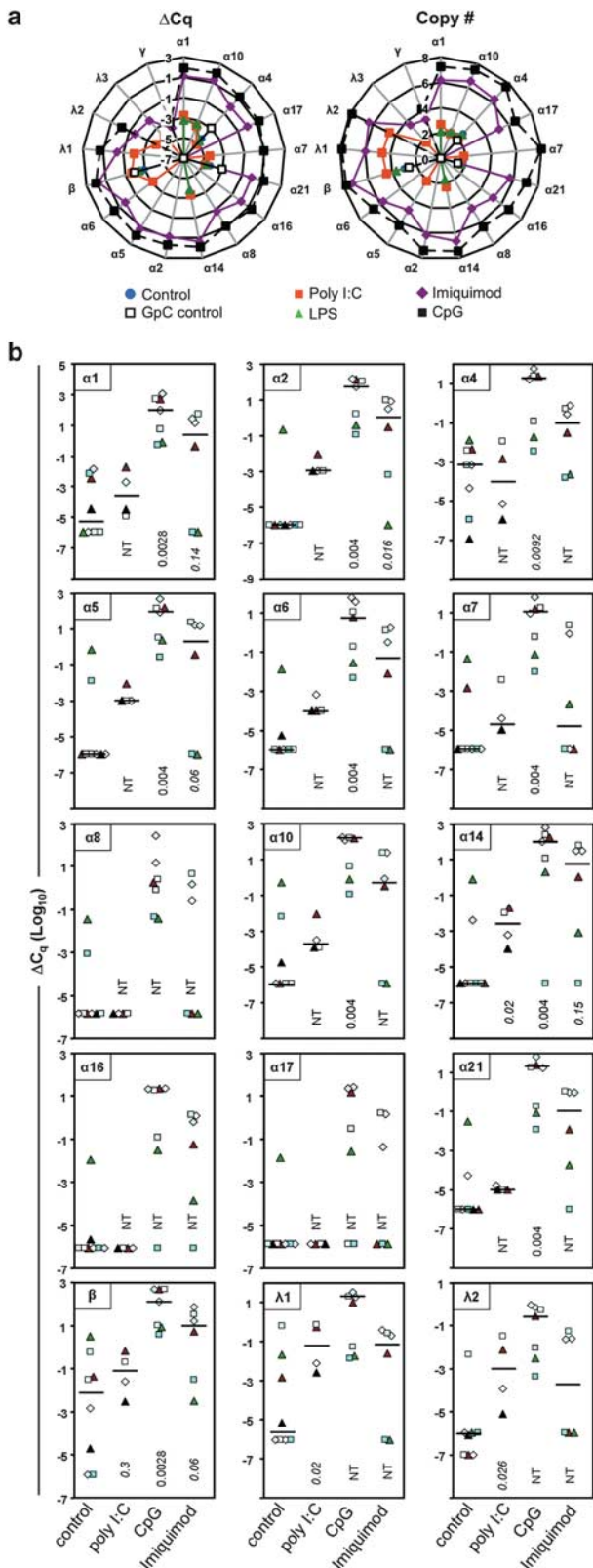
The format of the complete assay is a 384-well plate (Applied Biosystems) into which the primer/probe sets (5 μ l) are robotically distributed (Hudson Solo, Hudson Robotics, Springfield, NJ, USA), such that columns 1–5 are used for four-point standard curves derived from serial dilutions of cDNA for each subtype of IFN- α and IFN- λ , and for IFN- β and IFN- γ . Column 5 also includes

multiple wells for negative (that is, no added template) controls to rule out signal from primer dimers; those which consistently do not amplify are pooled. Columns 6 and 7 are used for measurement of expression of two HKG from each sample, and columns 8–24 are used for each of the IFN; such that 1 μ l

from the 21 μ l RT reaction was dispensed in each well across the row, allowing for 16 cDNA templates, one in each row, per plate (Supplementary Figure 3). For high-throughput analysis, once the primer/probe sets are distributed into the 384-well plates, they are dried with compressed air circulated through a heater set to 120 $^{\circ}$ C (Mechanical and Electrical Design Fabricators, NIH Office of Research Services, Bethesda, MD, USA) and directed through a 384 pin manifold (Zipvap 384, Glas-Col, LLC, Terre Haute, IN, USA) for ~60 min and stored in aluminum foil at 4 $^{\circ}$ C.

The volume of each PCR reaction for the data reported here was 5 μ l, consisting of 2.5 μ l of PCR Master Mix, 1.5 μ l of the primer/probe sets and 1 μ l of cDNA template. Plates were centrifuged briefly, the well contents mixed with a two-dimensional plate vortexer (MixMate, Eppendorf, Westbury, NY, USA) for 1 min at 2600 r.p.m., and then centrifuged again before qRT-PCR reaction (7900HT Fast Real-Time PCR System, Applied Biosystems). Data were analyzed with SDS Real-ware (version 2.3, Applied Biosystems) and then exported to an internally designed Microsoft Excel spreadsheet to calculate linearity (R^2) and efficiency of standard curves, copy number of each template expressed as a value per μ g RNA input, and ΔC_q for each of the two HKG, which are chosen according to the cell type as previously described.⁵¹

For precision analysis of the high-throughput assay, PCR master mix, cDNA templates for the four-point standard curves, and water (total volume 7.5 μ l) are mixed, and then distributed into the wells of the PCR plate with the Hudson Solo robot. The HKG primer/probe sets, diluted to 2.5 μ l, and then the sample cDNA (mixed with water and master mix to a total of 7.5 μ l) are added to each well with a Matrix multichannel pipette (Thermo-Fisher Scientific, Waltham, MA, USA).



IFN protein ELISA and gene expression of ISGs

Expression of IFN proteins was measured by ELISA (Verikine IFN- α multi subtype, IFN- β and IFN- λ (IL-28B/29/28A) kits, PBL Interferon Source) according to the manufacturer's instructions. Gene expression of *IRF7* and *MX1* was measured with pre-mixed primer/probe sets (Applied Biosystems) and ΔC_q calculated vs the HKG *UBC* (ubiquitin).

Toll-like receptor ligands

LPS (*Escherichia coli* O111:B4), poly I:C and imiquimod were purchased from EMD Chemicals (Gibbstown, NJ, USA), and were used at concentrations of 10 ng ml⁻¹, 25 μ g ml⁻¹ and 10 μ M, respectively. CpG type D35 (5'-GGTGCATC GATGCAGGGGGG-3') and control D35 GpC (5'-GGTGCATGCATG CAGGGGGG-3') oligonucleotides^{77,78} were synthesized by the FBR and were used at 1 μ M. For both oligonucleotides, the two bases at the 5' and 3' ends have nuclease-resistant phosphorothioate linkages.⁷⁷

Cells and cell culture

Buffy coats and elutriated monocytes (preparations that include mDC and pDC) were obtained from the NIH Clinical Center Department of Transfusion Medicine (Bethesda, MD, USA). PBMC were isolated from the buffy coats by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) density centrifugation and cultured at a concentration of 2.5×10^6 cells ml⁻¹. For three donors, the cells were stimulated immediately after isolation; for the other three, cells were stored in liquid nitrogen and after thawing, allowed to rest overnight before stimulation. All cells were cultured in RPMI 1640 medium with L-glutamine (Gibco, Carlsbad, CA, USA), 10% fetal bovine serum (Hyclone, Logan, UT,

Figure 5 Expression by pDC of all type I IFN in response to CpG. pDC were purified and stimulated with poly I:C, imiquimod, CpG or GpC control. (a) IFN expression is shown in log₁₀ scale as a function of expression of the HKG *SDHA* (ΔC_q , left) or copy number μ g⁻¹ RNA (right). Although all IFN- α subtypes are expressed in response to CpG, the pDC from this donor failed to express IFN- $\alpha 7$ in response to imiquimod. (b) Expression (ΔC_q) of IFN by seven donors and statistical analysis (with Bonferroni's correction) of types I and III IFN by pDC in response to CpG, imiquimod and poly I:C vs control as in Figure 4. Identical symbols in Figures 4 and 5 indicate that the cells are from the same donor.

USA), and 20 $\mu\text{g ml}^{-1}$ gentamicin (Invitrogen) and stimulated as recently reported.⁵⁶

From the elutriated cells, pDC, mDC and monocytes were serially purified in that order with magnetic beads specific for CD304, CD1c and CD14, respectively, using an AutoMACS magnetic cell sorter (Miltenyi Biotec, Auburn, CA, USA). Purity was validated by flow cytometry by staining each cell set with the combination of: PE-Texas Red anti-CD3 (clone UCHT1, Beckman-Coulter, Miami, FL, USA), Pacific Blue anti-CD20 (clone L27, kind gift of Mario Roederer), APC-Cy7 anti-CD14 (clone 63D3), Alexa-Fluor anti-HLA-DR (clone ivA12), fluorescein isothiocyanate anti-CD1c and PerCP-Cy5.5 anti-CD303 (BDCA-1 and BDCA-2, respectively, Miltenyi). Clones 63D3 and ivA12 were purified from mouse ascites fluid and conjugated to their respective fluorophores in-house.

Monocytes were cultured for 7 days in 100 ng ml^{-1} macrophage colony-stimulating factor (eBioscience, San Diego, CA, USA) to generate MDM, and 1000 U ml^{-1} of IL-4 (Peprotech, Rocky Hill, NJ, USA), 800 U ml^{-1} of granulocyte-macrophage colony-stimulating factor (Amgen, Thousand Oaks, CA, USA) and 0.05 mM β -mercaptoethanol (Sigma Aldrich) to generate MDCC.

Primary and *in vitro* differentiated cells were cultured at 1×10^6 cells ml^{-1} and stimulated with TLR ligands at the concentrations indicated for 4 h. Supernatants were harvested for measurement of secreted products, and cells were lysed using RLT buffer (Qiagen) for RNA purification and cDNA synthesis.

RNA isolation and cDNA synthesis

RNA was purified from cell lysates with Qiagen RNeasy spin columns and DNase digestion (Qiagen); concentration and purity ($\text{Abs}_{260/280}$) were measured with a NanoDrop 1000 spectrophotometer (Thomas Scientific, Waltham, MA, USA) and frozen at -80°C until use. Concentrations were ≥ 25 $\text{ng } \mu\text{l}^{-1}$ while $\text{A}_{260/280}$ ratios typically ranged from 1.95 to 2.05. First-strand cDNA synthesis was primed with both random hexamers and oligo-dT primers (Superscript III, Invitrogen) and up to 500 ng total RNA in 21 μl volume, followed by RNase H treatment according to manufacturer instructions.

Statistics

Comparison of IFN expression levels to control was tested for statistical significance after first visually assessing which IFN are expressed in response to a given stimulus. The values were then hierarchically tested from most to least likely to be significant, with the paired, non-parametric Wilcoxon signed-rank test (JMP software, Version 5, SAS Institute, Cary, NC, USA) until the *P*-value was >0.05 . Statistical significance for each panel of subtypes expressed in response to each ligand was determined using the Bonferroni correction. For example, for pDC responding to CpG, eleven IFN were tested for significance; therefore the threshold for significance for this set of tests is 0.05/11, or $P \leq 0.0045$. The sample collection and data analysis presented conform to the MIQE guidelines for publication of qRT-PCR experiments.⁷⁹

CONFLICT OF INTEREST

VPM and RLR are co-inventors of the technology to measure expression of IFN- α and IFN- λ subtypes and derive royalty income from it. PBL InterferonSource has licensed the technology to measure expression of IFN- α and IFN- λ subtypes and may derive income from it. The remaining authors declare no conflict of interest.

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Author contributions: VPM assisted in design and revision of primer/probe sets, and evaluated their performance. PH and VPM performed the expression experiments and contributed to the paper. LMS, ZZ, MBN, AC and SB assisted in primer/probe validation and

with the expression experiments. KDK optimized the qRT-PCR IFN assay for high-precision, high-throughput analysis, and performed many of the assays for the expression experiments. MP and DV contributed to experimental design. RGJ provided the cDNA for the IFN- α subtypes, advised experimental design and contributed to the paper. RLR designed the primer/probe sets and supervised the project. RLR wrote the paper.

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