Rigid Interferon- α Subtype Responses of Human Plasmacytoid Dendritic Cells

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The large family of human type I interferon (IFN) includes 13 distinct subtypes of IFN- α , all utilizing a single type I IFN receptor. Many viruses have created evasion strategies to disable this cytokine family, highlighting their importance in antiviral defense. It is unclear what advantage the presence of so many different IFN- α subtypes provides, but functional differences observed among individual IFN- α subtypes suggested that they might play distinct regulatory roles during an immune response. To determine whether IFN- α subtype responses differ depending on a particular type of insult and thus whether IFN- α subtype responses are flexible to adapt to distinct pathogen challenges, we developed a novel nested multiplex reverse transcriptase polymerase chain reaction assay with which we measured expression of all IFN- α subtypes by freshly isolated human plasmacytoid dendritic cells (pDCs), a main source of IFN- α following pathogen challenge. Collectively our data show a remarkable stability in the relative magnitude and the kinetics of induction for each IFN- α subtype produced by pDC. Although various stimuli used, A-, B- and C-class CpGs, live and heat-inactivated influenza viruses and the TLR7 agonist R837 affected the overall magnitude of the response, each IFN- α subtype was induced at statistically similar relative levels and with similar kinetics, thereby revealing a great degree of rigidity in the IFN- α response pattern of pDC. These data are most consistent with the induction of optimized ratios of IFN- α subtypes, each of which may have differing signaling properties or alternatively, a great degree of redundancy in the IFN- α response.

Introduction

TYPE I INTERFERONS (IFNs) have long been known as important effector cytokines with potent antiviral activity (Levy and others 2003; Theofilopoulos and others 2004). Many viruses have developed evasion strategies to circumvent or reduce the effects of IFN, highlighting the importance of this cytokine in antiviral defense (Gao and others 1997; Ronco and others 1998; Smith and others 2001; Talon and others 2000; Zhu and others 2002; Abate and others 2004; Loo and Gale 2007). It is increasingly recognized that these cytokines also play crucial immunomodulatory roles and affect both innate and adaptive immune responses that go well beyond their direct antiviral activity (Tough 2004; Aichele and others 2006; Banchereau and Pascual 2006; Coro and others 2006; Le Bon and others 2006; Chang and others 2007b). The mechanisms underlying this IFN-mediated immune response regulation, however, are incompletely understood. Given the fact that this cytokine is induced in

response to many different pathogens, both viral and bacterial, and that IFN expression and regulation are implicated as an underlying cause for at least some autoimmune diseases (Banchereau and Pascual 2006), a better understanding of type I IFN response regulation is of great importance.

Type I IFN constitutes a large family of acid-stable cytokines that in humans include IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω (Theofilopoulos and others 2004; Noppert and others 2007). Interferon- α itself is comprised of 13 subtypes that arose by gene duplication and show relatively minor (78–98% homology between the subtypes) but highly conserved sequence differences. Although the evolutionary advantage for generating or preserving so many different type I IFNs is unknown, data on functional differences of IFN- α subtypes might suggest that the differential induction of IFN- α subtypes could enable stimulus-dependent IFN-response modulation. Thus, distinct functional outcomes could be achieved depending on the type and nature

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of the stimulus, i.e., the type of pathogen a host is exposed to. Consistent with this hypothesis, a number of reports showed differences in the biological effects of individual IFN- α subtypes on leukocytes (Harada and others 1983; Hibbert and Foster 1999; Yanai and others 2001; Foster and others 2004; Ariyasu and others 2005). However, a comprehensive comparative analysis of all the pleiotropic functions of IFN- α has not been conducted to date, largely because of the difficulties studying these closely related gene products, and it is likely that not all effects of IFN have been identified. Overall, the antiproliferative and antiviral activities of IFN- α subtypes seem to be well correlated (van Pesch and others 2004), indicating large overlaps in the downstream signaling events regulating these distinct biological effects. The observed functional differences between individual IFN types and subtypes are attributed to differential binding to and/or signaling through the only known type I IFNR termed IFNAR (Aguet and others 1984; Meister and others 1986; Piehler and Schreiber 1999; Jaks and others 2007).

A certain hierarchy of expression of individual murine IFN- α subtypes was observed, which is regulated by the differential expression of various IFN regulatory factors (IRFs). Levy and others showed that virus-induced IFN- α 4 expression preceded expression of other IFN- α subtypes, because induction of this IFN- α subtype does not require IRF-7 synthesis (Marie and others 1998; Levy and others 2003). Instead, IFN- α 4 is transcriptionally induced by IRF3, which is constitutively expressed in many cell types and increased expression is induced by IFN- β . Once induced, IFN- α 4 will induce IRF-7 expression, which in turn will induce other IFN- α subtypes. This limited induction of a single IFN- α subtype might be the exception of IFN subtype regulation rather than the rule, as IRF-7 seems to broadly induce other IFN- α subtypes. However, whether it does so in a stepwise fashion has not been studied.

Plasmacytoid dendritic cells (pDCs) are a major IFN- α -producing subset of hematopoietic cells (Colonna and others 2004). These cells constitutively express IRF7, thus do not require IFN- α 4 induction for further IFN- α subset production, enabling them to rapidly and strongly produce IFN- α in response to virus infection (Izaguirre and others 2003; Prakash and others 2005). Furthermore, these cells have the capacity to retain certain triggers, such as A-class CpG's, in early endosomes for prolonged Toll-like receptor 9 (TLR9)-mediated IFN- α induction (Honda and others 2005; Lande and others 2007). IFN- α subtype response regulation by pDCs is of particular interest, as these cells migrate to regional lymph nodes following their activation and thus might be involved primarily in immune response regulation (Colonna and others 2004).

We conducted this study to determine whether human pDCs modulate the relative amounts of each IFN- α subset produced and thereby determine their ability to adapt to different pathogenic stimuli. A comprehensive analysis of all IFN- α subtypes expressed within the pDC population has not been conducted to date due to the difficulties in uniquely identifying and quantifying the highly homologous IFN- α subtypes. Analysis of cloned complementary DNA (cDNA) fragments of Sendai virus and herpes simplex virus (HSV)-stimulated myeloid and plasmacytoid DC indicated that

pDC can express a wider range of IFN- α subtypes compared to myeloid DC (Izaguirre and others 2003). In contrast, Coccia and others (2004) reported the induction of all IFN- α subtypes by both myeloid and plasmacytoid DC following exposure to influenza virus, albeit at greatly differing levels. This group used a SYBR Green approach to assay the different IFN- α subtypes. The generation of single-products was used as confirmation for assay specificity. Differences in the sensitivity of these two assays, cDNA cloning versus reverse transcriptase polymerase chain reaction (RT-PCR) might explain those apparent differences in their findings. Others compared IFN- α subtype expression of human peripheral blood mononuclear cell (PBMC) following infection with three different viruses (Loseke and others 2003). They used an approach similar to that used by us here (quantitative real-time RT-PCR) to analyze some but not all of the IFN- α subtypes (Loseke and others 2003). Similar to the studies by Coccia and others (2004), their results showed the induction of all measured IFN- α subtypes in virus-stimulated PBMC. Furthermore, they noted that the relative amounts of the individual IFN- α subtypes induced appeared independent of the type of virus used for stimulation but that there were also differences in the kinetics of expression. Because this study was conducted with whole PBMC, it is not clear whether differences in IFN- α subtypes might be due to differential stimulation of various DC populations, or stimulation of a different IFN- α subset profile within one cell population. By developing a highly sensitive two-step nested quantitative RT (qRT)-PCR approach to measure simultaneously all IFN- α subtypes in purified human pDC we determined that these cells express a remarkably stable IFN- α subtype response pattern. Although the magnitude of the IFN- α response was highly dependent on the type of stimulus used, the relative expression profile of the response remained unchanged.

Materials and Methods

Human subjects and isolation of pDC

Isolated leukocyte fractions (buffy coats) of human blood were obtained without donor identifiers from the Stanford Blood Center (Palo Alto, CA) following protocols approved by the UC Davis Internal Review Board on use of human subjects in research. PBMC from a total of nine donors were isolated by Ficoll-Paque (GE Healthcare, Piscataway, NJ) gradient centrifugation. For isolation of pDC from PBMC, cells were positively selected using the BDCA-4 Cell Isolation Kit and the autoMACS separator with the program recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). This separation method has been described as reducing IFN- α overall production in response to HSV exposure due to the inhibition of IRF-7 translocation to the nucleus (Fanning and others 2006). Thus, it is possible that the overall levels of IFN- α message in our experiments might be reduced due to the method of isolation. Due to the nature of the inhibition, it was not expected that this effect would be IFN-subtype specific. Aliquots of cells were stained with CD123, CD11c, and major histocompatibility complex class II (BD Biosciences, San Jose, CA) for analysis of purity using a FACSCalibur (BD Biosciences) for

data acquisition and FlowJo software (a kind gift from Adam Treestar, Tree Star, Ashland, Oregon) for data analysis. Purity of isolated pDC was >90%

Tissue culture and pDC stimulation

Freshly isolated pDC were plated at a concentration of 1×10^6 cells/mL (donors 1, 2, 8, and 9) or 5×10^5 cells/mL (donors 3–7) in 100 μ L of Rosewell Park Memorial Institute-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2-mercaptoethanol, and 10 mM HEPES (Invitrogen, Carlsbad, CA) in 96-well microtiter plates in the presence or absence of the following stimuli: A-Class CpG oligodeoxynucleotides (ODN) (50 μ g/mL; ODN 2336), B-Class CpG ODN (50 μ g/mL; ODN7909), C-Class CpG ODN (50 μ g/mL, ODN10101, or ODN10109) (Gu and others 2007) all kind gifts of Coley Pharmaceuticals, Ottawa, Canada), Imiquimod-R837 (5 μ g/mL; R837) (Invivogen, San Diego, CA), 0.5 or 5 multiplicity of infection (MOI) influenza virus A/Mem/71 or A/Puerto Rico/8 grown in embryonated hen eggs and evaluated as described (Doucett and others 2005). Virus was used either live or heat-inactivated by placing at 56°C for 30 min. For inhibition of IFN production 5 ng/mL of recombinant human or human cytomegalovirus interleukin-10 (IL-10) (R&D Systems, Minneapolis, MN) was added to the cultures concomitantly with pDC stimuli. Both types of IL-10 showed similar effects (Chang and others in preparation) and their biological effects are therefore referred to as “IL-10” for the purpose of this study.

IFN- α subtype cloning

Genomic sequences flanking the coding regions of the IFN- α subtypes were gathered from the NCBI sequence database and aligned using MultAlin (Corpet 1988). Subtype-specific PCR primers were chosen in areas that allowed each primer to incorporate as many differentiating nucleotides as possible (Table 1). At a minimum, the three prime terminal base of each primer was chosen to be unique to the targeted

subtype. Easy-A High-Fidelity PCR Cloning Enzyme (Agilent Technologies, Santa Clara, CA) was used to amplify all 13 IFN- α subtypes from Genomic DNA derived from human PBMCs. Amplicons were verified by agarose gel electrophoresis and inserted into plasmids using a pCR8/GW/TOPO TA Cloning Kit (Invitrogen). The resulting plasmids were sequenced and aligned to NCBI derived sequences.

RT-PCR for IFN- α subtype determination

To quantify transcription of all IFN- α subtypes, two-step nested multiplex real-time RT-PCR reactions were carried out with specific outer and inner primer and probe sets. Although we cloned both IFN- α 1 and IFN- α 13 to confirm efficient and similar amplification of both subtypes, these subtypes differ by only one amino acid. Therefore we chose primers and probes such that they were amplified and quantified together as IFN- α 1/13. RNA was extracted from single cell suspensions and processed as previously described (Baumgarth and others 2004; Dolganov and others 2001). Briefly, DNase-treated total RNA (2 ng/sample) was transcribed into cDNA using Superscript II (Promega, Madison, WI). An aliquot of the cDNA or control plasmid DNA was subjected to 15 rounds of a first-step multiplex preamplification PCR in the presence of 5 picomoles of each of the 12 specific outer primers (Fig. 1) in one 50 μ L reaction using Advantage 2 DNA Polymerase (TaKaRa Bio USA, Madison, WI). Additional outer primers for nine housekeeping genes (see below) were included (sequences provided upon request). The cycling parameters were as follows: (1) 94°C for 1 min; (2) (15 cycles: 94°C for 15 s; 55°C for 15 s; 70°C for 40 sec; (3) 70°C for 7 min.

For the second round of amplification, for each IFN subtype and housekeeping gene individual 10 μ L TaqMan assay reactions were set up in 384-well plates (Applied Biosystems, Foster City, CA) using only 0.004 μ L of preamplified product. This was important for minimizing carryover of nonused outer primers, which could result in subtype crossover. These reactions also contained 5 μ L 2 $\times$ BD QTAQ DNA Polymerase Mix (TaKaRa Bio USA, Madison, WI) and a set of subtype specific primers (300 nM each) and probe (100 nM) (Fig. 1).

TABLE 1. PCR PRIMER SEQUENCES FOR CLONING OF HUMAN IFN- α SUBTYPES

IFN- α subtype	Upstream primer (5'–3')	Downstream primer (5'–3')
1	ggctctaaactcatgtaagagtgcat	agcatgggtcatagttatagcaggg
2	taaacgagtagttccctatttaaggctag	aaaggtgagctggcatacga
4	caaaaacagagatagaaagtacaactaggga	tgctgcacaggtatacacaa
5	caaggttcagggtcactcaatct	agaatcatttcgatgttgaccagt
6	aactagtagttccctatttaagacacacat	cgtttctgtgttgatcaggtctt
7	catactttttatctgtgaagtagagggtatagtaga	attagtagaatgagaatcatttccatga
8	accagctcagcagcatcca	ggtctttgaaagagcacaactgt
10	ttcagaaaacctagaggccgaa	gcctgcacaggtatacacgaca
13	ggctctaaactcatgtaagagttcaag	tatcagcatgggtcatagttatagcagaa
14	ccagcagcatcttcgggatt	gtattagtagaatgagaatcatttccatga
16	gtctttgagtcaggggaaaaat	gaggatcatttccatgttgatgagt
17	caatgatgaaaaacaatgaaaaacattct	ggaggaaactcatgaaagtgtgagataa
21	agttttgagtcaggggaaaaat	aagtgtggactggtgtattagcaataa

FIG. 1. Primers and Probes for IFN- α subtype-specific cDNA amplification. Shown are the alignments of all human IFN- α subtypes with data obtained from the NCBI databank and alignment conducted using MultiAlign (Corpet 1988). Sequences identical to the consensus sequence are indicated by dots. Underlined are the (outer)-primers for the preamplification step and shaded in grey are the inner IFN- α subtype-specific primers. Note that probe sequences are shaded in black. Probes for IFN- α 2, 5, and 8 use antisense strand sequences, all others use sense strand sequences. Capitalized letters indicate that the sequence is identical to the consensus sequence but the primer base was modified to increase specificity. Bolded capitalized letters indicate sequence differences from consensus sequence with primer base modified to increase specificity.

Cycling parameters were as follows: (1) 95°C for 10 min; 2) 40 cycles: 95°C for 15 s, 57°C for 10s, 60°C for 1 min.

IFN- α ELISA

Supernatants of pDC cultures were taken and analyzed by enzyme-linked immunosorbent assay (ELISA) for total IFN- α and IFN- β using commercial kits according to the manufacturers instructions (PBL InterferonSource, Piscataway, NJ). The IFN- α Multi-subtype ELISA kit is specified to measure the following IFN subtypes: IFN1, 2, 4, 5, 6, 7, 8, 10, 16, and 17.

Data normalization and statistics

Raw cycle threshold numbers of amplified gene products were normalized to a housekeeping gene (ubiquitin

conjugating enzyme) to control for cDNA input amounts. This housekeeping gene was chosen as the most stably expressed gene from a total of nine housekeeping genes tested (elongation factor 1- α , peptidylprolyl isomerase A, metastatic lymph node 51, beta2 microglobulin, glyceraldehydes-3-phosphate dehydrogenase, ubiquitin, ribosomal protein L13a, hydroxymethylbilane synthase) as assessed using the GeNorm algorithm (www.wzw.tum.de/gene-quantification/) as outlined by others (Vandesompele and others 2002). Following normalization data were converted to relative copy numbers based on plasmid standard dilution curves created for each of the IFN- α subtypes.

A total of 66 different treatment/donor-combinations were analyzed. For each of these samples the natural log of absolute copy numbers for the 13 IFN subtypes were compared before and after stimulation with various agents. First, profile plots

were made for each treatment–experiment–donor combination. Then Tukey's one-degree-of-freedom test for additivity was performed ($p = 0.62$), indicating that the data follow an additive model with no significant interactions. Following that, a two-way analysis of variance (ANOVA) was fitted with the treatment strategy as one factor and the combination as the other to test for equality of mean copy numbers across treatment and across combination. Finally the residuals from the two-way ANOVA were examined for possible violation of the assumptions of normality and equal variances (Kutner and others 1996). All tests were two-sided at level 0.05; analyses and graphics were carried out using R (Team 2006).

Results

Development of a robust specific multiplex qRT-PCR-based method to detect individual IFN- α subtype genes

To determine the potential effects of differential type I IFN- α subtype responses of pDC we first developed an assay to quantify each IFN subtype. This presented two hurdles: first, only small quantities of RNA are available from freshly isolated human pDC for amplification and second, the sequences for the subtypes have very limited differences. Regarding the latter, we used a two-step multiplex qRT-PCR approach that we have previously described (Dolganov and others 2001; Baumgarth and others 2004), in which we first enrich target sequences in a quantitative fashion by running a multiplex PCR with outer primers potentially amplifying >100 genes in a single preamplification reaction, followed by a second-round of Taqman amplification (Dolganov and others 2001; Baumgarth and others 2004). We used SNIP-PCR-based strategies (i.e., deliberate introduction of mismatches into the primers and the use of short minor groove-binding probes that are sensitive to single nucleotide differences) to address the problem of high sequence homologies between individual IFN- α subtypes. Although single nucleotide differences have been used previously as distinguishing features in probes in quantitative assays (Kutyavin and others 2000; Ye and others 2001; de Kok and others 2002), we introduced mismatches in both primers and probes to achieve maximal subtype specificity.

The highly quantitative preamplification step is not intended to distinguish between subtypes but to amplify each subtype equally. Therefore, outer primers for all IFN- α subtypes were designed from a span of 169 nucleotides identified by aligning all IFN- α subtypes using MultAlin (Corpet 1988) that maximized the numbers of distinguishing nucleotide differences for all of the subtypes and was flanked by sequence containing relatively few nucleotide differences. Five forward and seven reverse preamplification (outer) primers were placed in the region of homologous sequences (Fig. 1) and the length of each primer was adjusted to achieve similar melting temperatures for all. These primers were pooled for use in a single reaction. To ensure equal amplification for all subtypes, titrations of genomic DNA (presence of a single copy per IFN- α subtype) and RNA for each individual IFN- α subtype were amplified using the pooled primers. For each doubling of DNA or RNA input the resulting amplification curves gave a slope of close to 1, showing a near-perfect linear

relationship between amplicon input and output (data not shown), similar to the results we have reported previously (Dolganov and others 2001; Baumgarth and others 2004).

Real-time (inner) PCR primers and probes were designed by selecting subtype-specific primers that incorporated as many naturally occurring subtype-distinguishing nucleotides as possible and for which one of the distinguishing nucleotides was positioned at its 3' terminus. The melting temperatures were adjusted by adjusting the length of individual primers. To ensure that these primers would not amplify nontargeted subtypes, additional mismatches were deliberately added 3 bases upstream from the end (–3 position) of some of the primers following rules that have been established for the amplification refractory mutation system procedure (Newton and others 1989; Little 1997): If the existing 3'-most base formed a weak mismatch (not necessarily PCR disabling) with the nontargeted subtypes, then a "strong," i.e., highly destabilizing mismatch was added at this –3 position. If the existing 3' terminal base formed a strong mismatch with the nontargeted subtypes, then a "weak" mismatch was added at the –3 position, and if a "moderate" strength mismatch was formed between the 3'-most base and the other subtype sequences, then a "moderate" strength mismatch was incorporated into the primer at the –3 position. When multiple mismatched bases were present in the nontargeted subtypes, the 3' terminal mismatch strength was determined from the nontargeted subtype base that gave the weakest mismatch. Conversely, the strength of the introduced mismatch was chosen to be at least strong enough to cover all of the subtypes. If a second mismatch already existed in the –3 position of the primer but was deemed inadequate, an introduced mismatch was either placed at an alternate, nearby site, or the second mismatch was strengthened by replacement with another base.

Primers and probe sets for each IFN-subtype were tested for their ability to detect the targeted subtype and discriminate nontargeted subtypes. Preamplified DNA was prepared from each of the 13 cloned IFN- α subtype control plasmids (Table 1). IFN- α 1 and IFN- α 13 were mixed in equal proportions and not distinguished at the amplification step. Each of the resulting 12 amplicons was used with each of the 12 TaqMan (inner) primer/probe sets to test for their ability to detect targeted and nontargeted subtypes. Table 2 shows that the approach resulted in an exquisitely specific assay that successfully distinguishes all IFN- α subtypes with the exception of IFN- α 1 and IFN- α 13, which differ by only one amino-acid sequence. Titrating genomic DNA assessed efficiency of amplification and assay sensitivity. The assay showed strong linearity and sensitivity with detection levels at 4 copies of DNA for all assays except IFN- α 8 (16 copies, Figure 2). Thus, this newly developed assay allows precise measurement of all individual human IFN- α subtypes in a single multiplex qRT-PCR reaction.

Activation of freshly isolated human pDCs results in stimulation-type dependent differences in the magnitude of the IFN response

In order to measure IFN- α subtype induction we first stimulated freshly isolated pDC from one donor with A, B,

FIG. 2. High sensitivity and linearity of amplification for all IFN- α subtypes. Shown are the cycle threshold numbers (C_t) obtained by Taqman amplification of indicated copy numbers of DNA preamplified from plasmids carrying individual cloned human IFN- α subtypes with primers and probes shown in Figure 1. Linear amplification was obtained with as little as four copies DNA for all but one IFN- α subtype (IFN- α 8), for which the limit of detection was determined to be 16 copies. Shown is one experiment of at least three performed for each subtype that gave similar results.

and C class CpG's, a control ODN, as well as two different replicating influenza virus strains: a human recombinant virus (A/Memphis/71) and a mouse-adapted strain (A/PR8) and measured the levels of induction of IFN- α protein and

each IFN-subtype messenger RNA (mRNA) after 16h (Fig. 3). The magnitude of the IFN response was strongly dependent on the type of stimulus provided to the pDC. As expected (Vollmer and others 2004), Class A CpG's induced the overall

TABLE 2. SPECIFICITY AND SENSITIVITY OF A qRT-PCR ASSAY THAT DISTINGUISHES ALL HUMAN IFN- α SUBTYPES

	Template subtype added (10^6 copies)											
	1 or 13	2	4	5	6	7	8	10	14	16	17	21
1 or 13	7.46											
2		6.94										
4			6.54									
5				6.93								
6					6.17			28.34				
7			26.46			7.51			28.19			
8							6.93					
10								6.62				
14						23.87	23.71	22.98	7.73		25.13	
16										6.97	26.47	
17											9.42	
21												8.64

Grey-filled cells: specific amplicon signal (C_t value).

Bold-italics: Value is below that calculated for one gene copy.

Blank: Value below threshold of detection (40 C_t).

strongest IFN- α protein response, followed by influenza A/Mem71. Interestingly, the mouse-adapted strain of influenza induced lower responses compared to the human isolate at the same MOI. This might indicate that the rate of infection correlates with the magnitude of the response. However, infection of cells with A/Mem71 at 10-fold lower MOI (0.5) resulted in somewhat higher mRNA levels for all IFN- α subtypes at 16h post infection (data not shown). In addition, exposure of pDC to live-virus, independent of the strain of influenza virus used, induced lower overall IFN- α responses compared to application of non-replicating virus (Table 3). Whether this is due to differences in the kinetics of the response, virus-induced cell death, or virus-induced inhibition of dsRNA via accumulation of the non-structural protein NS-1 (Talon and others 2000) will need further investigation.

Overall there was a strong correlation between the overall levels of IFN- α subtype mRNA induction and the levels of IFN- α protein secretion (Fig. 3A versus 3B). While the magnitude of induction differed between each of the IFN subtypes, whenever stimulation resulted in measurable protein secretion, all IFN- α subtypes were induced at the transcriptional level. The fact that A class CpG's induced the strongest protein response, while influenza induced stronger mRNA levels is explained by the time-point chosen for analysis of both protein and mRNA (16h). Consistent with reports by others (Coccia and others 2004), we found that peak mRNA induction following in vitro stimulation occurred around 6h. By 16h much of the mRNA levels are significantly reduced compared to earlier time points (Figure 4 and data not shown). We continued studying the 16h time point as a means to reliably measure both IFN- α protein as well as mRNA levels.

The B class CpG and the control ODN did not induce significant levels of IFN- α and both were omitted from further analyses. The concentration of B class CpG (50 μ g/mL) might have been suboptimal, as others reported moderate IFN- α induction by PBMC with doses 10-fold lower (Vollmer and others 2004). Importantly, independent of the stimulus used, those that induced IFN- α responses in pDC did so by inducing all IFN- α subtypes, albeit to differing levels. Similarly, stimulation of total PBMC with these and additional stimuli (soluble CD40L, LPS and poly (I:C)) either induced responses of all IFN- α subtypes or not at all. Overall those levels were, however, very low (data not shown).

Large donor-dependent differences in the magnitude but not the kinetics of IFN- α responses by human pDC

Further experiments were conducted with freshly isolated human pDC from eight additional donors. A total of 66 different treatment conditions and time points were analyzed (summarized in Table 3). Stimuli were used that resulted in robust IFN- α responses by pDC isolated from the first individual (Fig. 3). We aimed to determine whether stimulation of freshly isolated human pDC would show a differential pattern of IFN- α subtype induction that varied by time of measurement (6, 12, 16, and 18h), the type of stimuli used, or by pDC donor. Culture supernatants were assessed for the presence of IFN- α protein and RNA was extracted for

each culture to measure relative copy numbers for each of the IFN- α subtypes.

Similar to the previous experiments, there was an excellent correlation between the levels of IFN- α transcript induction in pDC and the levels of total IFN protein measured in the supernatants. For each condition that resulted in measurable IFN-induction at the protein level, all of the IFN-subtypes were induced at the transcriptional level. However, for each donor, each stimulation condition caused distinct levels of IFN- α secretion (Table 3 and Figure 4A) and mRNA expression (Figures 4B and 5). The differences were quite marked, with ranges of protein concentrations differing up to 80-fold between cultures of donors stimulated under similar conditions (Table 3).

However, there did not appear to be any obvious differences in the kinetics of the IFN- α subset induction by pDC at the mRNA level (Figure 4B and data not shown). All stimulation conditions chosen induced the highest levels of mRNA expression at the 6-h time point, independent on the IFN- α subset studied. At the 12- and 18- time points all IFN- α subset genes were reduced. However the degree of reduction was dependent on the type of stimulus used (Fig. 4B). This was reflected in the IFN- α protein levels. A- and C-class CpG's stimulation resulted in different levels of protein production and mRNA expression mainly at the later time points studied. Both classes of CpG induced similar levels of IFN- α secretion at 6 h. While A-class CpG-induced IFN- α protein continued to accumulate in the supernatant (Fig. 4A) and levels of mRNA remained relatively high (Fig. 4B), IFN- α protein levels triggered by C-class CpG's remained unchanged after the 6-h time point and mRNA levels were considerable lower at the 12 and 18-h time points, indicating that most cytokines were produced within the first 6 h (Fig. 4A). This is consistent with the known early endosomal retention of A-class but not C-class CpG's that continue to trigger TLR9-mediated signals for the induction of IFN- α for a prolonged period (Honda and others 2005; Lande and others 2007).

Activation of freshly isolated human pDCs results in a similar rigid pattern of IFN subtype induction independent of stimuli or donor origin

Somewhat surprisingly, analysis of all IFN- α subtypes showed that there was a distinct pattern of mRNA subtype induction by pDCs that appeared independent of the time point of the analysis, the overall magnitude of the response or the donor. Each subtype appeared to be induced to the same degree relative to all other IFN- α subtypes (Fig. 4B). To determine whether this pattern of induction observed with CpG stimulation was specific to the triggering of the TLR9 pathway, we stimulated pDC with influenza virus. Influenza virus does not engage TLR9 but rather signals through TLR7 and RIG-I (Diebold and others 2004; Lund and others 2004; Kato and others 2006; Pichlmair and others 2006). Stimulation of pDC with both live and heat-inactivated influenza virus also induced the same pattern of IFN- α subtypes that was induced with all classes of CpG that resulted in measurable IFN responses (Fig. 4B). Stimulation with the TLR7 agonist R837 resulted in only small levels of IFN- α protein and mRNA induction (Table 3), but the overall

TABLE 3. IFN- α PROTEIN (ng/mL) SECRETION BY HUMAN pDC IN RESPONSE TO TLR AGONISTS

Treatment ^a	No stimulus	A-class CpG		C-class CpG (10101)	C-class CpG (10109)	Flu virus Live ^b	Flu virus inactive		R837	
		–	+	–	–	–	–	+	–	+
IL-10 ^c	–	–	+	–	–	–	–	+	–	+
Donor										
1	0.021	2,000	140	320	310	1,400				
2	0.004	810	39		220	180	390			
3		25	8.1				28	11		
4		64	5.1				33	10		
5		76	19				87	7.2	3.5	0.7
6		39	8.3						0.7	0.2
7		100	9.6						0.7	0.2
8		79		78	100	310	520			
9		750		170	190	380	390			

^aSupernatants were collected from individual cultures at 16 h poststimulation except for donors 8 and 9. For those cultures pDC were stimulated for 6, 12, and 18 h. The 18-h data are provided here for comparison and results from all time points are shown in Figure 4A. ^bFor donor 1, live and inactivated influenza virus was applied to cultures at an MOI of 0.5 and 5. Shown are data for MOI of 5. ^cpDC cultures were treated with (+) or without (–) 5 ng/mL of recombinant human IL-10 upon stimulation.

profile of the mRNA induction was again very similar to that induced with the TLR9 agonists and influenza virus (data not shown).

We next compared gene expression for all pDC samples treated under the conditions summarized in Table 3 to determine the robustness of this emerging IFN- α mRNA subset expression pattern. Graphic representation (Fig. 5) indicated a strong similarity in relative levels of IFN- α subtype gene expression for all IFN- α subtypes independent of the magnitude of the response or the type of stimulus used. Statistical analysis of all 792 total measurements (66 different samples/stimulation conditions from a total of 9 pDC donors $\times$ 12 measurements for all IFN- α subsets for each sample) confirmed this finding. Comparison of the natural logarithm of the copy number counts showed that an additive model best fitted the data generated. Thus, there was no significant deviation from an emerging pDC pattern of IFN- α subset expression ($p = 0.62$). With exception of some samples in which levels of IFN were very low (particularly those of IL-10-stimulated cultures, Fig. 5B), all treatment conditions independent of donor origin, type of stimuli or time of analysis following culture set-up, gave a similar induction pattern.

Weak induction of IFN- $\alpha 6$ in human pDC

Most IFN- α subtype genes were induced at similar copy number levels, varying at most 5- to 10-fold. The notable

exception was IFN- $\alpha 6$. This gene was consistently expressed at levels 10- to 50-fold lower compared to the other IFN subtypes (Fig. 5). However, this gene was clearly induced by the stimuli used here and thus does not appear to be a pseudogene, as suggested earlier (Hiscott and others 1984; Lopez and others 1997). Additional technical controls were conducted, including use of alternate primers and probe, which confirmed that the sensitivity of the assay for IFN- $\alpha 6$ was similar to that of all other subtypes (Fig. 1 and data not shown). Thus, IFN- $\alpha 6$ gene expression is significantly lower compared to all other IFN- α subtypes, possibly due to the presence of silencer elements within its promoter region (Lopez and others 1997).

Broad inhibition of all IFN- α subtypes in the presence of IL-10

IL-10 is known to inhibit multiple functions of antigen-presenting cells, including maturation, migration, survival, and various effector functions, such as secretion of cytokines (Moore and others 2001; Chang and others 2004; Chang and others 2007a). IL-10 is also known to inhibit overall IFN- α production and mRNA expression of human virus-stimulated PBMC (Payvandi and others 1998) as well as total IFN- α protein production by isolated pDC (Duramad and others 2003). We therefore sought to determine its effects on the individual IFN- α subsets. Consistent with published

FIG. 3. pDC respond to different innate stimuli with differential induction of IFN- α . **(A)** Purified human pDC were left untreated or were stimulated with indicated classes of CpG's at previously determined optimal concentrations and with replicating influenza A virus (two strains: A/Mem71, a human isolate and A/PR8, a mouse-adapted virus) for 16 h. IFN- α production was determined by ELISA on culture supernatants. Data are mean levels from cultures set up in duplicates. Untreated and control CpG-treated cultures did not contain measurable amounts of IFN- α . **(B)** Cells were harvested following culture and RNA extracted for determination of IFN- α subtype induction. Shown are fold-changes in gene expression compared to the untreated cells. Shown are representative results from one of at least three conducted with individual donor pDC.

reports, addition of IL-10 to cultures of freshly isolated pDC stimulated with various agonists strongly reduced the secretion of IFN- α (Table 3). Analysis at the gene expression level showed that the inhibition of IL-10 affected all IFN- α subtypes similarly. IL-10 reduced the overall levels of IFN- α expression while preserving the distinct "pattern" of relative IFN- α subtype responses elaborated by pDC (Fig. 5B). Thus, it did not modify pDC-mediated IFN responses by regulating individual IFN- α subset contributions.

In summary, the data presented here demonstrate the surprisingly rigid response pattern of human pDC with regard to individual IFN- α subtypes. While the overall magnitude of the IFN- α responses differed markedly depending on the type of stimulus used, the patterns of induction of individual IFN- α subtypes or the levels of their repression by IL-10 were remarkably stable.

Discussion

The presence of many IFN- α subtypes in the genome of most species, 13 in the case of humans, all signaling through a single receptor has long created an enigma. What is the evolutionary advantage of establishing or maintaining this large number of IFN genes? Are these genes individually regulated to provide immune cells with the flexibility to fine-tune particular type I IFN responses, depending on the type of pathogen or type of insult inducing the response? Thus, are there many different profiles of IFN- α responses? Data presented here now demonstrate that for one of the major leukocytic sources of IFN- α , the pDC, the answer appears to be "no." While human pDC strongly regulated the magnitude of their type I IFN response depending on the type of stimulus provided, the relative abundance of each IFN- α subtype produced, i.e., the quality of the IFN- α response, remained unchanged. Moreover IL-10, a strongly immunomodulatory cytokine that is known to suppress induction of pDC maturation and function, suppressed IFN- α secretion by reducing all IFN- α subtypes to the same degree. Thus, for a given cell type (pDC) the subtype IFN- α response pattern seems remarkably fixed.

Over the last decade an increasing number of reports have provided a detailed analysis of host cell transcriptional responses to various pathogenic stimuli (reviewed in Jenner and Young 2005). Those studies have identified cell-type specific gene transcriptional response profiles that are common between various triggers, stimuli such as pathogens as diverse as yeast and viruses. On the other hand, individual

cell types also showed the ability to induce subsets of genes in a highly stimulus-specific fashion (Huang and others 2001; Boldrick and others 2002; Nau and others 2002). The type I IFN-induced response pattern (IFN-effector response) is one of the dominant common transcriptional patterns induced by both viruses and certain bacteria, further highlighting the importance of this cytokine system in immune defense and regulation. Because of the high sequence homology between individual IFN- α subtypes, current microarray technology does not usually distinguish individual IFN- α subsets and systematic information on qualitative and quantitative IFN- α subset responses has therefore mainly been lacking. In contrast to a number of previous studies (Izaguirre and others 2003; Loseke and others 2003; Coccia and others 2004; Fung and others 2004; Palmer and others 2007), our approach studied the effects of various known IFN triggers on a highly purified cell population and capturing all IFN- α subsets in a highly quantitative fashion. We thereby excluded variations in IFN- α subset production by different cell subsets and measurement sensitivity issues from our analysis. The technology outlined here builds on our previous work (Dolganov and others 2001; Baumgarth and others 2004) in which we developed a highly quantitative multiplex assay that relies on the use of gene-specific primers during the initial preamplification step to avoid read-out bias and to accurately measure gene induction in small sample sizes. The application of this assay described here provides a rapid, highly quantitative and relatively straightforward novel approach to studying all IFN genes (other IFN genes such as IFN- κ , IFN- β , and others can easily be added to the analysis, data not shown) while requiring very small quantities of starting materials.

Signaling through the TLR pathways can induce both common and specific transcriptional responses (Jenner and Young 2005). It appears from our studies that stimulation of pDC with two distinct and potent stimuli for IFN- α production that engage distinct TLR-signaling pathways, namely on one hand TLR7 (Diebold and others 2004; Lund and others 2004) and potentially RIG-I (Kato and others 2006; Pichlmair and others 2006) following influenza virus infection and on the other TLR9 via CpG stimulation, induce all IFN- α subtypes as part of a common transcriptional pDC response. These genes are switched on or off, with each being induced or reduced (by IL-10) at levels that are fixed in relation to each other. Our findings of the induction of all IFN- α subtypes by these stimuli are consistent with and expand a recent study by Coccia and others (2004). Our analysis

FIG. 4. Differences in magnitude but not kinetics of IFN- α following pDC stimulation with various stimuli. (A) Freshly isolated pDC from two individual donors were activated as indicated with various stimuli and IFN- α protein levels were determined in supernatants of cultures at 6 h (white), 12 h (grey), and 18 h (black) postculture onset by ELISA. Shown are mean levels IFN- α set up in duplicate. (B) Quantitative RT-PCR was conducted on all cell cultured for which supernatants were collected to assess the kinetics of all IFN- α subtypes induced in pDC at various times after infection. Shown are examples of gene expression profiles for all IFN- α genes of donor #8 following strong (A-class CpG, *top panel*) and weak (C-class CpG, *middle panel*) induction via TLR9 and TLR7/RIG-I by live (solid lines, *bottom panel*) or heat-inactivated (dashed lines, *bottom panel*) influenza virus. Note that while the magnitude of mRNA induction differed at the different time points and between the different stimuli used, the kinetics of induction and the relative levels by which each subtype was induced were similar.

of IFN- α induction by pDC was not exhaustive, leaving the possibility that IFN- α subtype responses might be adjusted in response to pathogens not used here, and/or by the stimulation of other signaling pathways. Alternatively, it is also possible that certain viruses use interference with this very static IFN- α response by pDC as an immune evasion strategy. Nonetheless, we show that in response to two major TLR-pathways (TLR7 and TLR9) that are engaged in response to many and very diverse sets of pathogens, the outcomes are remarkably similar.

It is interesting that the main adjustment in the IFN- α response by pDC appears to be in the magnitude rather than the quality of induction. Consistent with a global effect of a mix of different type I IFN- α proteins that are regulated by common pathways, rather than modified in their overall function by the regulation of individual subtypes, stimulation of pDC in the presence of viral (CMV) or human IL-10 reduced IFN- α levels to a similar extent (Fig. 5B), thus even in its inhibition maintaining the pattern of IFN- α gene

expression levels, albeit at a greatly reduced level. From a purely practical standpoint this means that measurement of any of the IFN subtypes, possibly with exception of the only weakly induced IFN- α 6 (Fig. 5), can provide a good indication of the overall IFN- α response. The question arises whether studies on the functionality of individual IFN- α subsets are useful for our understanding of IFN-mediated immune protection, given that IFN- α subtypes are not secreted alone, but always in conjunction with all other IFN- α subtypes and that assessment of concentrations in solid tissues is challenging. Isolation of particular cocktails of IFN- α subtypes as produced by individual cell subsets might better reveal their effects. In this context it will be important also to study cell types other than pDC to determine whether each cell type differs with regard to its particular IFN- α signature pattern. Plasmacytoid DC seem unusual in that they are able to rapidly induce IFN- α , without prior induction of IRF7 by IRF-3/IFN α 4 (Izaguirre and others 2003; Prakash and others 2005). However, when we attempted to measure IFN- α induction by freshly isolated macrophages and epithelial cell cultures, we saw, as expected, much weaker overall induction of IFN- α genes and stronger induction of IFN- β , but for cultures for which we saw measurable IFN- α induction, their relative levels appeared similar to those induced by pDC (data not shown).

The differences between A-class and C-class CpG's in their ability to induce different levels of IFN- α responses by pDC are well appreciated and the mechanisms are thought to involve the kinetics of endosomal degradation of various TLR9 agonists (Rothenfusser and others 2002; Honda and others 2005; Lande and others 2007). Our studies now show that the prolonged triggering of TLR9 by A-class CpG in early endosomes induces more, but not qualitatively different subtypes of IFN- α . The IFN- α subtype distribution between A- and C-class CpG's were the same at all time points studied (Fig. 4). Thus, any IFN-mediated functional differences induced by these CpG's at least in pDC are not due to differences in the IFN- α subtype distribution, but

FIG. 5. Induction of a rigid IFN- α subtype transcriptional response pattern by pDC. (A) Freshly isolated pDC from nine individual donors were activated under a total of 66 different stimulation conditions, as summarized in Table 3. Shown are IFN- α gene copy numbers (natural log) for each IFN- α subtype as determined by qRT-PCR for all conditions. Lines in bold indicate copy numbers of nonstimulated pDC. While the overall magnitude of the IFN- α subtype response varied greatly between the different stimulation conditions and donors, the induction pattern, i.e., the degree to which each subtype was induced was very similar for all cultures, showing no statistically significant differences ($p = 0.62$) of induction. Statistical analysis was done as outlined under Materials and Methods. (B) Similarly, treatment of pDC with IL-10 in the presence of various stimuli reduced the overall levels of IFN- α secreted (Table 3) and the transcriptional induction of each IFN- α subtype to the same degree, as shown here.

rather a reflection of the duration and magnitude of the response. This should provide valuable information for the further exploitation of CpG's as potential therapeutic agents by removing one potential variable to their effects.

In summary, this study has identified an IFN- α signature subset response pattern by pDC that is modulated in its magnitude but not its quality through triggering of individual signaling pathways. TLR9 agonists, such as A-class CpG, as well as influenza virus (presumably through engagement of TLR7 and RIG-I) induce strong and rapid IFN- α responses of a similar quality. While IL-10 affects the magnitude of the response it does not alter this pattern, providing further indication that the major regulation of IFN- α responses is at the level of its induction. The assay we developed will now allow a systematic assessment of IFN- α subset response qualities induced in response to other pathogens and by other cell types to determine how representative studies on pDC are for our understanding of this complex and potent cytokine family.

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