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# Self-priming determines high type I IFN production by plasmacytoid dendritic cells

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## Abstract

Plasmacytoid dendritic cells (pDCs) are responsible for the robust and immediate production of type I IFNs during viral infection. pDCs employ TLR7 and TLR9 to detect RNA and CpG motifs present in microbial genomes. CpG-A was the first synthetic stimulus available that induced large amounts of IFN-α (type I IFN) in pDCs. CpG-B, however, only weakly activates pDCs to produce IFN-α. Here, we demonstrate that differences in the kinetics of TLR9 activation in human pDCs are essential for the understanding of the functional difference between CpG-A and CpG-B. While CpG-B quickly induces IFN-α production in pDCs, CpG-A stimulation results in delayed yet maximal IFN-α induction. Constitutive production of low levels of type I IFN in pDCs, acting in a paracrine and autocrine fashion, turned out to be the key mechanism responsible for this phenomenon. At high cell density, pDC-derived, constitutive type I IFN production primes pDCs for maximal TLR responsiveness. This accounts for the high activity of higher structured TLR agonists that trigger type I IFN production in a delayed fashion. Altogether, these data demonstrate that high type I IFN production by pDCs cannot be simply ascribed to cell-autonomous mechanisms, yet critically depends on the local immune context.

### Keywords

CpG-A; CpG-B; TLR9; pDC; Type I IFN

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## Introduction

The recognition of invading pathogens and the initiation of an appropriate immune response are key functions of the innate immune system. TLRs are a family of PRRs that sense conserved microbial signatures, also known as PAMPs [1, 2]. Among these, TLR9 detects unmethylated CpG-motifs, which are present in viral and bacterial DNA, whereas TLR7 senses uridine-rich RNA. To prevent recognition of endogenous nucleic acids, both TLR7 and TLR9 are confined to endosomal compartments that are usually devoid of nucleic acids. While both TLR7 and TLR9 are broadly expressed in innate immune cells of the murine system, both TLR7 and TLR9 show a far more restricted expression pattern within the human system, with TLR9 expression restricted to B cells and pDCs.

TLR9 traffics from the endoplasmic reticulum through the Golgi apparatus to acidic endosomal compartments, where it is proteolytically cleaved to achieve its signaling-competent state. Following ligand engagement, TLR9 initiates a signaling cascade, which is dependent on MyD88. In pDCs, MyD88 triggers a signaling complex consisting of IRAK1/4, TRAF3, and IKKα that leads to phosphorylation and nuclear translocation of interferon regulatory factor 7 (IRF7) and transcription of type I IFN genes. At the same time, TLR9 engagement activates NF-κB and subsequent proinflammatory gene expression [3].

For TLR9, different classes of synthetic CpG-oligodeoxynucleotides (ODNs) have been described [4, 5]. Class A CpG-ODNs (CpG-A) induce large amounts of type I IFN in pDCs, whereas CpG-B is a weak interferon inducer but triggers maturation of pDCs and B cells. We have previously shown that CpG-A forms nucleic acid-based nanoparticles in the size range of viruses, whereas CpG-B ODNs are only present in their monomeric state [6]. Overcoming this conformation by complexation with nanoparticles allows CpG-B to induce maximal type I interferon induction in pDCs comparable to that of CpG-A.

The exceptional capacity of pDCs to produce large amounts of type I IFNs in response to certain ligands has mainly been explained as a cell-intrinsic phenomenon that is operational at the single-cell level. To this effect, several models have been proposed in which the subcellular localization of receptor engagement and its subsequent signaling plays an important role. In the so-called spatio-temporal model of TLR signaling, the specific ability of pDCs to produce high amounts of type I IFN is attributed to the unique functionality of pDCs to retain certain TLR ligands within the early endosomal compartment where a prolonged activation of the MyD88/IRF7 signaling complex results in high type I IFN production [7, 8]. In these studies, it has been shown that CpG-A is retained within the early endosome for a prolonged period, whereas CpG-B is rapidly transferred to endolysosomes where IRF7 activation is only minimal. More recently, two further models of spatial regulation of TLR9 signal transduction have been put forth to explain the robust type I IFN response of pDCs. In this regard, it has been shown that certain TLR9 ligands (e.g. CpG-A) are translocated from early endosomes via an AP3-dependent membrane trafficking pathway into an acidic LAMP-$2^{+}$ lysosome-related organelle from which IRF7 signaling is initiated [9]. Of note, in this study, early endosomes ($VAMP3^{+}$) were shown to trigger NF-κB activation only. Furthermore, it was reported that pDC-dependent type I IFN production can also occur in an AP3-independent fashion. As such it was shown that certain DNA ligands

(DNA-immune complexes) can be translocated by LC3-associated phagocytosis into a LAMP-1$^+$ phagolysosome from which robust IRF7 activation emanates [10]. In the latter two activation models, a second signal (AP3 activation or initiation of LC3-associated phagocytosis) determines the formation of a specialized signaling compartment, from which TLR9 can initiate type I IFN production. NF-κB activation, on the other hand, proceeds as the default response from the early endosome or early phagosome. As a consequence, these models explain the differential activity of distinct TLR9 ligands (e.g. CpG-A or CpG-B) on the basis of their capacity to activate such a second signal.

Here, we show that the differential activity of distinct TLR9 or TLR7 ligands is mainly determined by their different kinetics in triggering TLR activation. Monomeric TLR ligands (CpG-B or R848) initiate rapid signal transduction, whereas more highly structured TLR ligands (CpG-A or complexed RNA) lead to delayed receptor activation. pDCs constitutively produce small amounts of type I IFNs, which results in a strong feedforward regulation of their TLR-triggered type I IFN production in vitro. As a consequence, delayed TLR activators such as CpG-A stimulate far higher type I IFN responses, given the fact that pDCs have been preprimed in an autocrine or paracrine fashion upon prolonged in vitro culture.

## Results

### TLR ligands differ in their capacity for and kinetics of type I interferon induction in pDCs

As previously reported, CpG-A is a strong stimulus for TLR9-dependent induction of IFN-α in pDCs, whereas CpG-B results in a comparatively weak IFN-α response. In addition to the difference in magnitude, a marked divergence in the kinetics of IFN-α production was observed (Fig. 1A and B) [11]. CpG-B-mediated IFN-α induction could be detected within the first 2–8 h following stimulation, whereas most of the IFN-α induced by CpG-A was not seen until 20 h after stimulation. Nanoparticle formation of CpG-B via complexation with polycationic peptides was able to overcome the comparatively low induction of type I interferon via CpG-B. Compared with the monomeric agonist CpG-B, nanoparticle complexed CpG-B resulted in a strong increase in IFN-α induction with delayed kinetics. Similar results were obtained when the monomeric TLR7-ligand R848 was compared with a more highly structured TLR7-agonist (polycationic peptide-complexed RNA). R848 induced up to 2 ng/mL IFN-α within the first 4 h, whereas the more highly structured TLR7-agonist RNA resulted in a 20-fold higher, yet delayed, induction of IFN-α in pDCs (Supporting Information Fig. 1A and B). Interestingly, when we added CpG-B simultaneously with CpG-A, type I IFN production was as low as CpG-B stimulation itself (Fig. 1C). Indeed, the later CpG-B was added to CpG-A-stimulated pDCs, the smaller the inhibitory activity turned out. At the same time, combining CpG-B and R848 did not synergize in terms of type I IFN production (Supporting Information Fig. 1C). Altogether, these results indicate that high IFN-α production is associated with delayed IFN-α induction and that CpG-B exerts a dominant effect over CpG-A stimulation in pDCs.

### Preincubation with supernatant of stimulated pDCs increases IFN-α production by pDCs via type I IFN

To test whether high, yet delayed, type I interferon induction upon CpG-A stimulation requires de novo protein translation, we stimulated pDCs with CpG-A or CpG-B in the presence or absence of the translation inhibitor cycloheximide and studied transcription of IFN-α and IFN-β upon activation at their peak levels (Fig. 2A and Supporting Information Fig. 2A). Whereas cycloheximide almost completely blocked IFN-α and IFN-β gene expression following CpG-A stimulation, CpG-B-mediated type I IFN induction was only partially affected. As such, we hypothesized that the high IFN-α induction elicited by CpG-A would depend on a de novo induced soluble factor acting in a paracrine or autocrine manner. To address this hypothesis, we designed experiments in which we transferred supernatant from pDCs that had been incubated with various TLR-ligands for 24 h to freshly isolated pDCs that were then stimulated for another period of 24 h. As expected, in unprimed, freshly isolated pDC, CpG-A clearly surpassed CpG-B in its IFN-α induction (Fig. 2B, i). On the other hand, when CpG-A-conditioned medium was used to prime freshly isolated pDCs, a robust level of type I IFN in the supernatants was already seen in the absence of additional stimulation (Fig. 2B, ii). This baseline level could be ascribed to IFN-α that was already present in the transferred supernatant and not due to de novo induction by the supernatant itself (data not shown). Nevertheless, under these conditions, both CpG-B and CpG-A elicited similar amounts of IFN-α. Moreover, a strong synergistic boost in IFN-α induction was seen when supernatant from CpG-B-stimulated pDCs was added onto freshly isolated pDCs that were stimulated with CpG-B thereafter (Fig. 2B, iii). Indeed, under these conditions CpG-B induced IFN-α within the same range as CpG-A, even though supernatant from CpG-B-prestimulated pDCs did not trigger type I IFN itself. Interestingly, CpG-A stimulation had only a marginal benefit over transfer of supernatant from prestimulated pDC cultures.

We considered various mechanisms that could account for the transfer phenomenon leading to strong interferon production by CpG-B. Among these, type I IFN seemed to be the most likely, given the fact that IFNAR signaling was critically important for maximal type I IFN production by CpG-A itself (Supporting Information Fig. 2B) [11]. To address this hypothesis, we first conducted experiments in which we blocked the biological activity of type I IFN cytokines. Indeed, a complete inhibition of the observed phenomenon was seen when antibodies against the type I interferon receptor were used (Fig. 2C). Consistent with this finding, a similar effect could be elicited when recombinant IFN-β was used instead of supernatant from CpG-B-stimulated pDCs. Priming pDCs with recombinant IFN-β led to a strong boost in type I IFN production upon CpG-B or R848 stimulation, whereas IFN-β by itself did not induce IFN-α. Of note, approximately 250 U/mL IFN-β had to be added at least 3 h prior to exert maximal priming activity (Fig. 2D and E, and Supporting Information Fig. 2C and D). Given these results, it appears unlikely that direct activation of components of the signal transduction pathway (e.g. phosphorylation of STAT1) is sufficient for the observed priming effect. Rather, de novo protein synthesis appears to be required to exert full priming activity. Indeed, in the presence of the translation inhibitor cycloheximide, the priming activity of recombinant IFN-β was completely abrogated (Supporting Information Fig. 2E). Moreover, as observed for the supernatant transfer experiments, IFN-β priming of

pDCs was not able to enhance the activity of CpG-A with regards to type I IFN production (Fig. 2F). Altogether, these results indicate that type I IFNs within the supernatant of CpG-B-stimulated cells exert a strong synergistic effect on monomeric TLR ligands on pDCs.

## Constitutive type I IFN production primes IFN-α responses in pDCs upon subsequent TLR stimulation

To our surprise, when control experiments were conducted using supernatant of unstimulated pDCs, a similar picture was seen as with supernatants from preactivated pDCs. Indeed, medium from unstimulated pDCs boosted the response of freshly isolated pDCs toward CpG-B just as well as supernatant from prestimulated pDCs and again this phenomenon turned out to be IFNAR dependent (Fig. 3A and Supporting Information Fig. 3A). This priming effect was pDC-specific, as supernatant from other cell types was not able to induce the observed synergy (data not shown). Indeed as little as 0.625 vol% supernatant from unstimulated pDCs still had remarkable activity in terms of boosting the IFN-α response upon CpG-B stimulation (Fig. 3B). These data suggested that in vitro cultured pDCs spontaneously produce type I IFNs in the absence of TLR ligation. To address this question, we made use of a reporter cell line, in which the type I IFN-inducible ISG54 promoter drives the expression of a reporter construct (Fig. 3C). Supernatant from pDCs that had been cultured for 24 h and then transferred onto this reporter cell line elicited a strong ISRE response within the range of 800 U/mL IFN-β, whereas supernatant from cultured monocytes elicited no ISRE activation (Supporting Information Fig. 3B). To address whether supernatant of unstimulated, in vitro cultured pDCs exerted antiviral activity, we incubated A549 cells with supernatant from pDCs or monocytes and subsequently infected the cells with EMCV. As little as 1 vol% of the supernatant from cultured pDCs protected cells from EMCV whereas supernatant from cultured monocytes showed no protective activity (Fig. 3D). Quantitative measurement of cell survival revealed that as little as 0.25 vol% supernatant from pDCs were as protective as 1 U/mL recombinant IFN-α (Supporting Information Fig. 3C). However, even though supernatant of unstimulated pDCs showed significant antiviral activity, neither IFN-α nor IFN-β could be measured at the protein level by ELISA in this supernatant (data not shown). Yet when we analyzed type I and III IFN mRNA expression by real-time PCR, low levels of IFN-α, IFN-β, and IFN-λ2 were detectable after prolonged in vitro culture (Fig. 3E). This spontaneous type I and type III IFN production was critically dependent on cell density. IFN-α, IFN-β, and IFN-λ2 expression, as well as ISRE-dependent expression (STAT1, IRF7, and IP-10) showed a strong decline with a decreasing number of pDCs in culture, whereas control transcripts (HPRT1 and BDCA2) showed no change in expression (Fig. 3F). In addition, constitutive type I IFN production by pDCs was sensitive to NF-κB and AP1 inhibition, whereas the immediate, PRR-dependent type I IFN production was not blunted when blocking these pathways (Supporting Information Fig. 4). Altogether these results indicate that cultured pDCs induce biologically active type I IFNs at high cell density in the absence of stimulation and that this constitutive induction is able to induce ISRE expression upon prolonged culture.

### Preincubation of unstimulated pDCs strongly primes towards higher IFN-α production

The above results indicated that supernatant of medium-incubated pDCs strongly activates the IFNAR-axis, thereby priming supernatant-treated pDCs for a strong type I IFN response. This prompted us to address the impact of prolonged incubation on the induction of IFN-α by subsequent TLR ligation. A series of experiments was conducted in which pDCs were incubated with medium for 24 hand subsequently stimulated with either CpG-A, CpG-B, or R848 for an additional period of 36 h. As expected, a marked difference in terms of IFN-α production was seen for CpG-A, CpG-B, and R848 when pDCs were directly stimulated without prolonged preincubation (Fig. 4A, solid bars). However, when pDCs were preincubated for 24 h prior to stimulation, both CpG-B and R848 induced similar amounts of IFN-α as CpG-A (Fig. 4A, hatched bars). Indeed, preincubation for various time periods prior to stimulation resulted in a gradual increase of more than six- and 11-fold in terms of IFN-α production for CpG-B and R848, respectively (Fig. 4B). Blocking type I IFN activity strongly inhibited the effect of prolonged in vitro culture, indicating that spontaneous type I IFN production was responsible for this effect (Fig. 4C).

Given our previous results on the role of high cell density for the spontaneous type I IFN response of pDCs, we hypothesized that the high type I IFN production following CpG-A stimulation was also dependent on the cell density at the time of stimulation. To address this hypothesis, we studied the activity of the different TLR ligands at different cell concentrations. Purified pDCs were plated at a density of 25 000 cells per well in 96-well plates and then downtitrated in five serial dilutions (dilution factor of 1:1) to 780 cells per well and subsequently stimulated with the different TLR ligands for 36 h. As expected, CpG-A induced more IFN-α at the highest cell density, whereas both R848 and CpG-B had considerably lower IFN-α induction activity. However, with declining cell number, the amount of CpG-A-induced IFN-α production per cell gradually decreased, whereas IFN-α production per cell for both R848 and CpG-B was not subject to cell density effects (Fig. 4D and E). At the lowest cell number analyzed (780 cells per well), almost no difference in IFN-α production for the tested TLR-ligands could be seen. Altogether these data provided clear evidence that pDC-derived spontaneous type I IFN production primes pDCs in an autocrine and paracrine fashion to strongly boost a subsequent TLR-triggered IFN response. In addition, these data suggest that this self-priming activity is critically required in order for delayed TLR activators to trigger their maximal IFN response.

## Discussion

Studying the different activities of pDC-stimulating TLR ligands, we have uncovered a novel feedforward mechanism that helps to explain the unique type I IFN-producing capacity of pDCs (Fig. 5). At a critical cell density, pDCs constitutively produce low amounts of type I IFN, which results in a positive feedforward regulation of their TLR-triggered type I IFN response. Under in vitro conditions, this positive regulation has a tremendous impact on the activity of different types of TLR ligands that differ in their kinetics of triggering pDC activation. Monomeric stimuli, such as R848 or CpG-B, initiate a rapid activation of their respective receptors and signaling cascades, with type I IFN production seen as early as 2–4 h after stimulation. Under these conditions, pDCs are not

preprimed by their auto/paracrine feedforward loop and thus type I IFN production is not amplified. On the other hand, TLR stimuli presented in a particulate form trigger pDC activation in a delayed fashion. CpG-A, for example, constitutes a self-aggregating DNA nanoparticle that gains access to lysosomal compartments only late in the course of stimulation, depending on an AP3-dependent translocation pathway. Consequently, initiation of TLR9 activation is delayed when pDCs have been primed by their spontaneous type I IFN feedforward loop, resulting in a strong type I IFN response. This delayed activation also explains why CpG-A-dependent type I IFN production requires de novo protein synthesis, whereas CpG-B-driven pDC activation represents an immediate event that does not depend on additional proteins being synthesized. In line with these observations, CpG-B can be converted into a strong type I IFN inducer by adding recombinant IFN-β, which substitutes for the spontaneous self-priming loop in vitro. At the same time, prolonged in vitro culture results in self-priming of pDCs and thereby converts rapid but weak activators into high type I IFN inducers. Consistent with this notion, blocking IFNAR signaling in pDC markedly impacts CpG-A-mediated type I IFN production, whereas CpG-B-triggered type I IFN production is not affected [11]. Of note, we interpret this phenomenon as a disruption of the spontaneous type I IFN feedforward loop and not as an inhibition of a positive feedback regulation. Interestingly, once activated via TLR9, type I IFN can no longer amplify the type I IFN response. This also explains why concomitant addition of CpG-A and CpG-B does not result in CpG-A-like activation of pDCs, but keeps type I IFN production at the level of CpG-B as seen in this study and in PBMCs in a previous study [12]. The mechanisms of refractoriness to further stimulation remains unclear, yet it can be speculated that CpG-B prestimulation limits ligand availability for another stimulus or that it impacts on the signaling cascade itself, e.g. by inducing negative feedback mechanisms. Altogether, these results unravel the critical role of spontaneous, low level type I IFN production in self-priming of pDCs toward maximal type I IFN production. Moreover, these data suggest that high type I IFN production by pDCs cannot be explained at the single cell level, yet that it is critically dependent on factors that operate in trans as part of the local immune environment.

Our study shows that a critical density of pDCs is required to initiate the spontaneous type I IFN response during in vitro culture. Indeed, it has long been known that purified pDCs form large cell clusters upon prolonged culture in vitro [13]. Our data imply that this cluster formation is critically required to initiate spontaneous priming. At the same time, it has been shown in vivo that activated human and also murine pDCs are mainly present as large cell clusters in T-cell rich areas of secondary lymphoid organs [14, 15]. As such, we assume that pDC accumulation in lymphoid tissue represents the physiological correlate of the in vitro observed cluster formation, thereby providing the prerequisite for the positive type I IFN feedforward loop described here. This is in line with reports demonstrating that type I IFN activity is critical for murine pDC activation in vitro and in vivo [16, 17], while pDCs are capable of an IFNAR-independent, early, yet weak IFN-α response [18]. However, it has also been noted that other studies have reported that pDCs do not require functional IFNAR signaling to exert type I IFN production themselves [19, 20]. Of note, these conflicting results might be due to different stimulation models studied, differences in time points analyzed and also different modes of type I IFN detection being employed.

Consequently, these data have important implications for the interpretation of in vitro studies involving pDCs and the in vivo application of pDC activating TLR ligands. Based on the model proposed here, the in vitro activity of a TLR ligand does not necessarily correspond to its in vivo activity, if unprimed pDCs are used. Indeed, various studies have shown that in vitro activities of different TLR9 ligands are only poor predictors for their in vivo activity. For example, CpG-B class oligonucleotides were shown to display high IFN-α inducing activity in mice and primates in vivo, even though hardly any type I IFN response was observed in vitro [21]. We speculate that this big difference in in vitro versus in vivo activity is mainly due to the fact that pDCs are preprimed in vivo, whereas freshly isolated, blood-derived pDCs are priming naïve.

It has been known for a long time that certain cell types produce type I IFN at a constitutive level and that this activity plays an important regulatory role for many biological processes. Even though it is difficult to conclude from early reports whether spontaneous I IFN production was actually studied under sterile conditions, several recent studies have unequivocally shown that certain cell types indeed produce low, but significant, levels of type I IFNs in the absence of infection. This phenomenon of tonic type I IFN production has been observed in mouse embryonic fibroblasts (MEFs) [22], myeloid dendritic cells [23, 24], osteoclast precursors, and also in pDCs [25], where it is has been shown to modulate cell differentiation and immune responses. While the exact triggering mechanisms for spontaneous type I IFN production remain elusive for most cell types, it has been shown for osteoclast precursor cells that RANKL is responsible for the low, but constitute expression of IFN-β. Here, IFN-β production acts as an autoregulatory mechanism controlling bone homeostasis [26]. Furthermore, basal levels of constitutive type I IFN have been shown to maintain and mobilize the HSC niche thereby regulating HSC homeostasis [27, 28]. The regulation of constitutively induced type I IFN at the transcriptional level has mainly been studied in fibroblast cultures, in which it is clearly distinct from pathogen-induced IFN. While IRF3 and IRF7 are instrumental for PRR-triggered type I IFN production, they are dispensable for tonic type I IFN production [22, 29]. On the other hand, AP-1 and NF-kB components are critically required for spontaneous type I IFN production, while being important, but not necessary, for pathogen-triggered IFN production [26, 29-31]. In analogy to these reports, our data show that spontaneous type I IFN production in pDCs also requires NF-kB and AP-1, whereas PRR-triggered type I IFN production is not majorly affected upon NF-kB or AP-1 inhibition. We speculate that a growth factor or cytokine-dependent signaling cascade is responsible for NF-kB/AP-1 dependent tonic type I IFN production. Of note, another important source of tonic type I IFN production can origin via PRR-dependent mechanisms in myeloid cells of the gut. In this regard, it has been shown that commensal microbiota provide an important source of PRR ligands under steady state that are important to facilitate antiviral immunity [32-34]. While we can exclude this route of paracrine priming in our in vitro assays, this mode of action might be of relevance for optimal pDC responses in vivo.

The natural capacity of pDCs to produce high amounts of type I IFN has been ascribed to the constitutive expression of IRF7 in pDCs [35]. It has been proposed that this endogenous IRF7 expression is critical for IFNAR-independent rapid interferon production. However, IRF7 has a very short half-life and must be continuously produced for its amplification

function [36, 37] and as an ISRE, the induction of IRF7 expression requires the activity of type I IFNs. As such, the enhanced pDC response following exposure to type I IFNs could be due to the enhanced expression of IRF7. In line with this notion, we have observed that exposure of freshly isolated pDCs to recombinant IFN-β or supernatant from in vitro cultured pDCs led to a marked increase in IRF7 expression (Supporting Information Fig. 4B). Consequently, based on previous work [25] and our own data, we suggest that tonic type I IFN production in pDCs controls the high level of IRF7 expression, which represents a critical factor determining the high type I IFN producing capacity of pDCs.

Of note, our data are not in contrast to previously published data, yet they suggest an alternative interpretation of the spatio-temporal signaling model. Indeed, in our model, the subcellular trafficking of CpG-A or analogous stimuli mainly determines high type I IFN responses due to delayed access to signaling-competent compartments and thus the activation of a pDC that had been subject to type I IFN priming. Consequently, we observe that type I IFN production by pDCs cannot simply be explained at the cellular or subcellular level. Indeed, potent type I IFN-inducing TLR agonists lose their activity at low cell density, where no self-priming is observed. Altogether, these data help to explain the long-known puzzle of different TLR agonists inducing low or high type I IFN responses in pDCs. Moreover, these data have important implications for the development and assessment of pDC-directed TLR agonists or antagonists for clinical application.

## Materials and Methods

### Media and reagents

RPMI 1640 (Biochrom) supplemented with 10% heat-inactivated FCS (Invitrogen), 1 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all Sigma-Aldrich), and 10 ng/mL IL-3 (Peprotech) or 10 ng/mL GM-CSF (ImmunoTools) was used. Recombinant human IFN-β was from Peprotech. Completely and partially phosphorothioate-modified ODNs were from Metabion. Small letters indicate phosphorothioate linkage, whereas capital letters stand for phosphodiester linkage 3′ of the base: ODN 2006: 5′-tcgtcgttttgtcgttttgtcgtt-3′; ODN 2216: 5′-ggGGGACGATCGTCgggggG-3′. R848 was from Invivogen. The single-stranded RNA oligonucleotide RNA9.2sense: 5′-AGCUUAACCUGUCCUUCAA-3′ [38] was from Eurogentec. ssRNA and DNA oligonucleotides were complexed with poly-l-arginine as previously described [39]. For blocking experiments, the neutralizing anti-IFN-receptor chain 2 antibody (PBL Biomedical Laboratories) was employed. pDCs were preincubated with this antibody or a respective isotype control (mouse IgG2a) at 10 μg/mL for 30 min prior to stimulation. To inhibit protein synthesis, pDCs were treated with cycloheximide (Sigma-Aldrich) 30 min prior to stimulation. Bay 11–7082 and SR 11302 were from Tocris Bioscience. The IFN-α ELISA was from Bender MedSystems.

### Cell culture

Freshly prepared buffy coats from human donors were obtained from the Institute for Experimental Hematology and Transfusion Medicine, University Hospital of Bonn with the

donors' written informed consent. The work with human cells was approved by the institutional review board.

PBMCs were obtained by Ficoll–Hypaque density gradient centrifugation. pDCs were isolated by MACS using the BCDA-4 dendritic cell isolation kit (Miltenyi Biotec) as previously described [39]. The purity of isolated pDCs (BDCA2-positive and CD123-positive cells) was above 95%. If not otherwise indicated, pDCs were cultured in 96-well round bottom wells at a density of 25 000 cells/well in 100 μL complete medium supplemented with IL-3, whereas monocytes were cultured in GM-CSF.

### Flow cytometry

Flow cytometric data were acquired on a FACSCalibur flow cytometer (BD Biosciences). Human pDCs were identified by positive staining with anti-BDCA2, anti-CD123 PE, and anti-MHC II PerCP and negative staining with anti-lineage FITC (BD Biosciences).

### Quantitative real-time RT-PCR

Real-time RT-PCR was performed as previously described [39]. Primer sequences are available on request.

### Type I IFN bioassay

HEK-Blue IFN-α/β cells (InvivoGen) were used as reporter cells, which allow the detection of bioactive human type I IFNs. This cell line expresses secreted embryonic alkaline phosphatase under the control of the IFN-α/β inducible ISG54 promoter.

### Viral Protection Assay

Where indicated A549 cells were preincubated with supernatant from in vitro cultured pDCs or monocytes for 18 h followed by infection with EMCV (a gift from W. Barchet) at a MOI of 1 for 18 h. Cell survival was determined by the CellTiter-Blue Cell Viability Assay (Promega). Cells were then fixed in 3.7% paraformaldehyde and stained with 0.05% crystal violet. Microscopy was performed on a Zeiss Axio Observer.Z1.

### Statistical analysis

Statistical significance was assessed using a Student's t-test for paired samples, whereas differences with $*p < 0.05$ were considered statistically significant, and those with $**p < 0.01$ were considered highly statistically significant. Statistical analyses were performed using GraphPad Prism (Version 5, GraphPad Software, Inc., La Jolla, USA).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
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| **pDC** | plasmacytoid dendritic cell |
| **IRF7** | interferon regulatory factor 7 |

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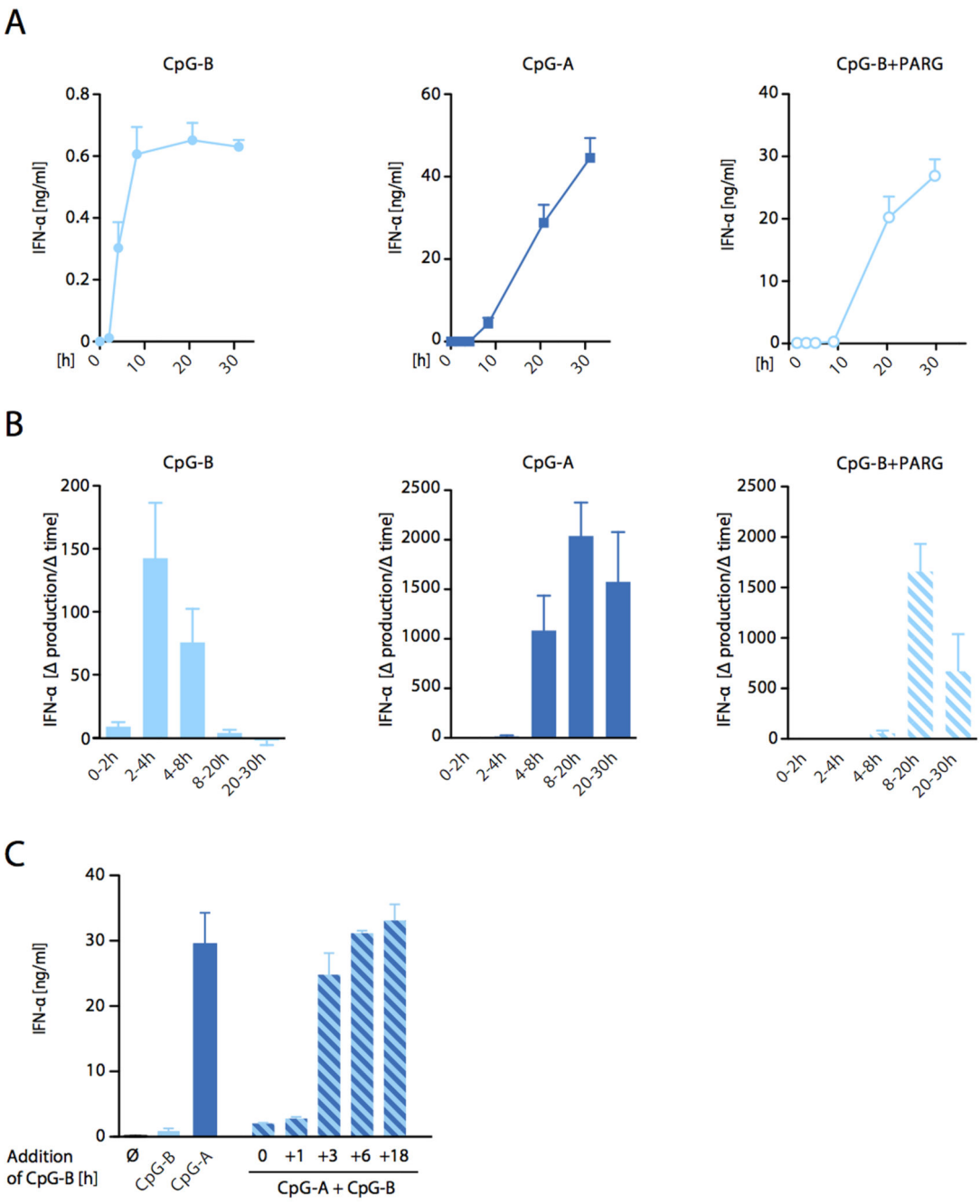


**Figure 1.**

TLR ligands differ in their capacity for and kinetics of IFN-α induction in plasmacytoid dendritic cells. (A) pDCs were isolated from PBMCs and cultured in 96-well plates (25 000 cells/well) with CpG-A (ODN 2216) or monomeric CpG-B (ODN 2006) (3 μg/mL) or complexed CpG-B. Supernatants were collected after the indicated periods and IFN-α was measured by ELISA. Mean IFN-α production at 2/4/8 h was not detectable (n.d.)/80/4370 pg/mL for CpG-A and n.d./n.d./218 pg/mL for complexed CpG-B, respectively. (B) The increase in IFN-α production over time (in pg/mL) was analyzed by dividing the increase in

IFN-α production by the time span (in h) of the period analyzed. (C) pDCs were stimulated with CpG-A and CpG-B was added at the indicated time points (hatched bars). In addition, untreated, CpG-B, or CpG-A only treated pDCs were included as controls (full bars). Supernatants were collected after 36 h and IFN-α was measured by ELISA. In (A–C), data from four independent experiments were pooled and are shown as mean values + SEM of four samples.



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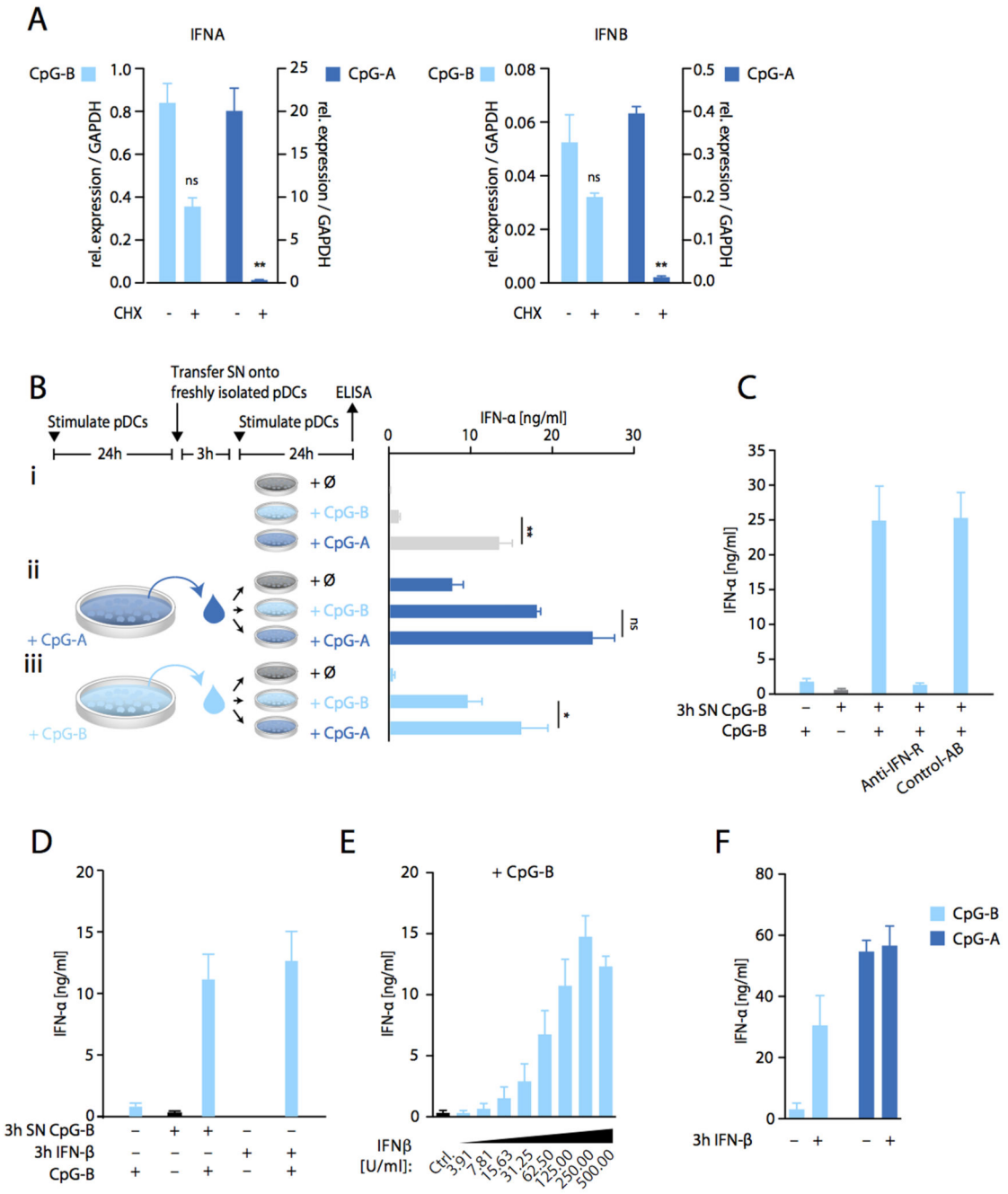


**Figure 2.**

Preincubation with supernatant of stimulated pDCs increases IFN-α production by pDCs via type I IFN. (A) pDCs (100 000 cells/well) were stimulated with CpG-A or CpG-B in the presence or absence of cycloheximide (CHX; 100 μM). After 3 h (CpG-B) and 12 h (CpG-A) cells were harvested and mRNA was isolated. Subsequently, IFN-α and IFN-β mRNA expression was analyzed by real-time RT-PCR using GAPDH for normalization. Results are shown as mean + SEM of four samples pooled from four independent experiments. (B) pDCs were stimulated with CpG-A or CpG-B for 24 h. Supernatants (SN) were collected

and added to freshly isolated pDCs. After 3 h, CpG-A, CpG-B, or medium was added for 24 h. Supernatants were collected after 36 h and IFN-α was measured by ELISA. Results are shown as mean + SEM of three samples pooled from three independent experiments. (C) pDCs were primed with SN from CpG-B-stimulated pDCs followed by CpG-B treatment in the presence or absence of a blocking antibody against the type I IFN receptor. Supernatants were collected after 36 h and IFN-α was measured by ELISA. Results are shown as mean + SEM of four samples pooled from four independent experiments. (D) pDCs were primed with SN from CpG-B-stimulated pDCs or recombinant IFN-β. Then cells were stimulated with CpG-B. Supernatants were collected after 36 h and IFN-α was measured by ELISA. Results are shown as mean + SEM of four samples pooled from four independent experiments. (E) pDCs were preincubated with the indicated amounts of recombinant IFN-β for 3 h followed by CpG-B treatment. Supernatants were collected after 36 h and IFN-α was measured by ELISA. (F) pDCs were primed with 250 IU/mL IFN-β for 3 h and then stimulated with CpG-A or CpG-B. Results for (E–F) are shown as mean + SEM of three samples pooled from three independent experiments. *$p < 0.05$, **$p < 0.01$ (paired Student's t-test).



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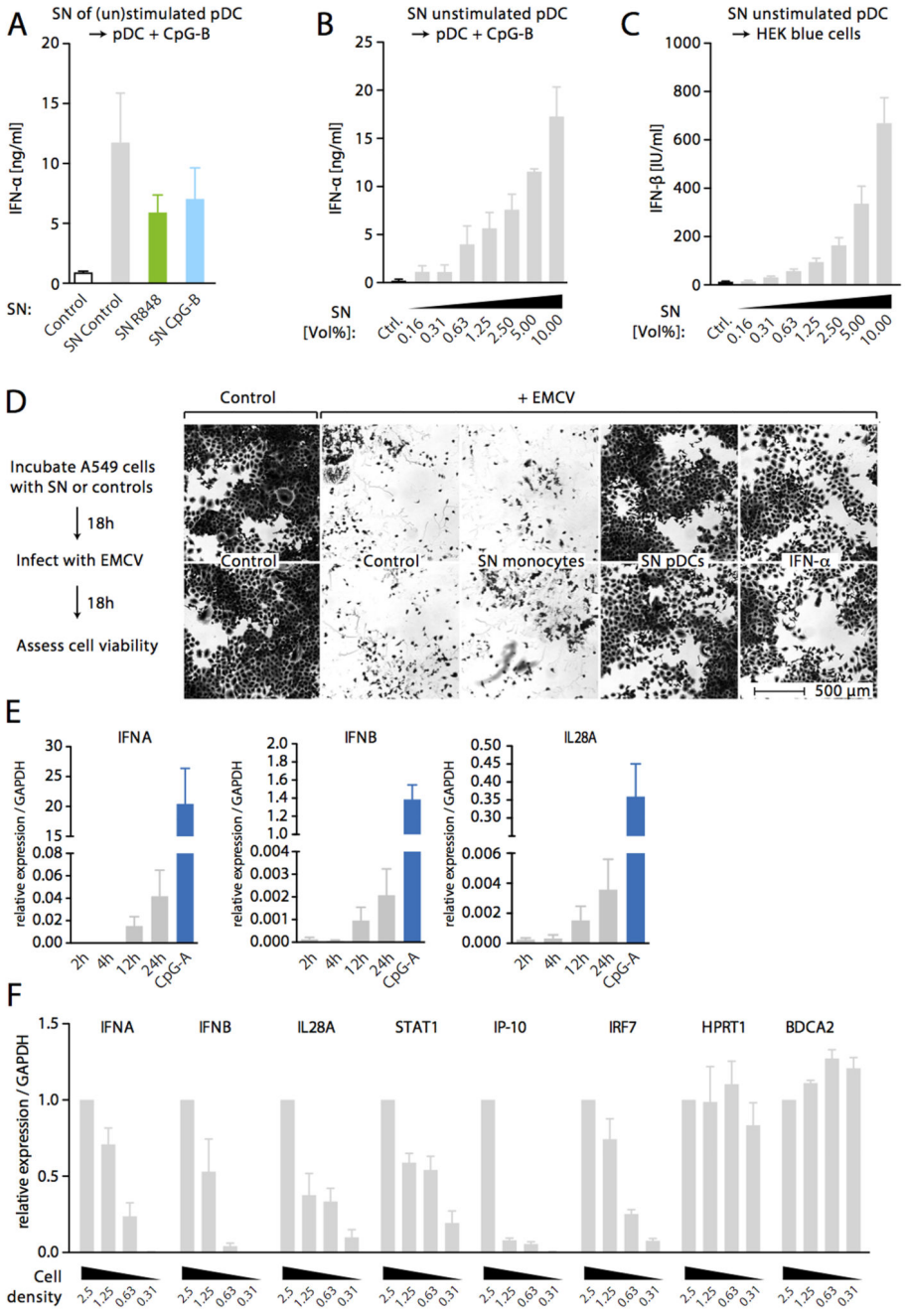


**Figure 3.**

Constitutive type I IFN production primes IFN-α responses in pDCs upon subsequent TLR-stimulation. (A) SN from pDCs that were incubated with CpG-B, R848, or medium for 24 h were collected and added to freshly isolated pDCs. After 3 h, pDCs were stimulated with CpG-B. IFN-α was measured after 36 h by ELISA. Results are shown as mean + SEM of five samples pooled from five independent experiments. (B) SN from medium-incubated pDCs were added onto freshly isolated pDCs at the indicated ratios. IFN-α was measured after 36 h by ELISA. Results are shown as mean + SEM of three samples pooled from three

independent experiments. (C) SN of pDCs incubated in medium for 24 h were tested for the presence of type I IFN (IU/mL) using HEK-blue human IFN-α/β reporter cells. Results are shown as mean + SEM of four samples pooled from four independent experiments. (D) A549 cells were either left untreated or primed with 1 vol% SN of monocytes, 1 vol% SN of pDCs or 1 IU/mL recombinant IFN-α for 18 h followed by EMCV infection. After additional 18 h cells were stained with crystal violet and imaged using bright field microscopy (2.5× objective). Two independent visual fields per condition are depicted and data are representative of two independent experiments. (E) pDCs (100 000 cells/well) were incubated in medium for the indicated periods or stimulated with CpG-A for 12 h. mRNA-levels of IFN-α, IFN-β, IFN-λ2 (IL28A) were analyzed by real-time RT-PCR. GAPDH mRNA expression was used for normalization. Results are shown as mean + SEM of four samples pooled from four independent experiments. (F) pDCs were titrated starting at 2 500 000 cells/mL with serial dilutions (1:1) down to 312 500 cells/mL. After 24 h mRNA expression was assessed by quantitative RT-PCR and normalized to GAPDH mRNA levels. Results are shown as mean + SEM of three samples pooled from three independent experiments.



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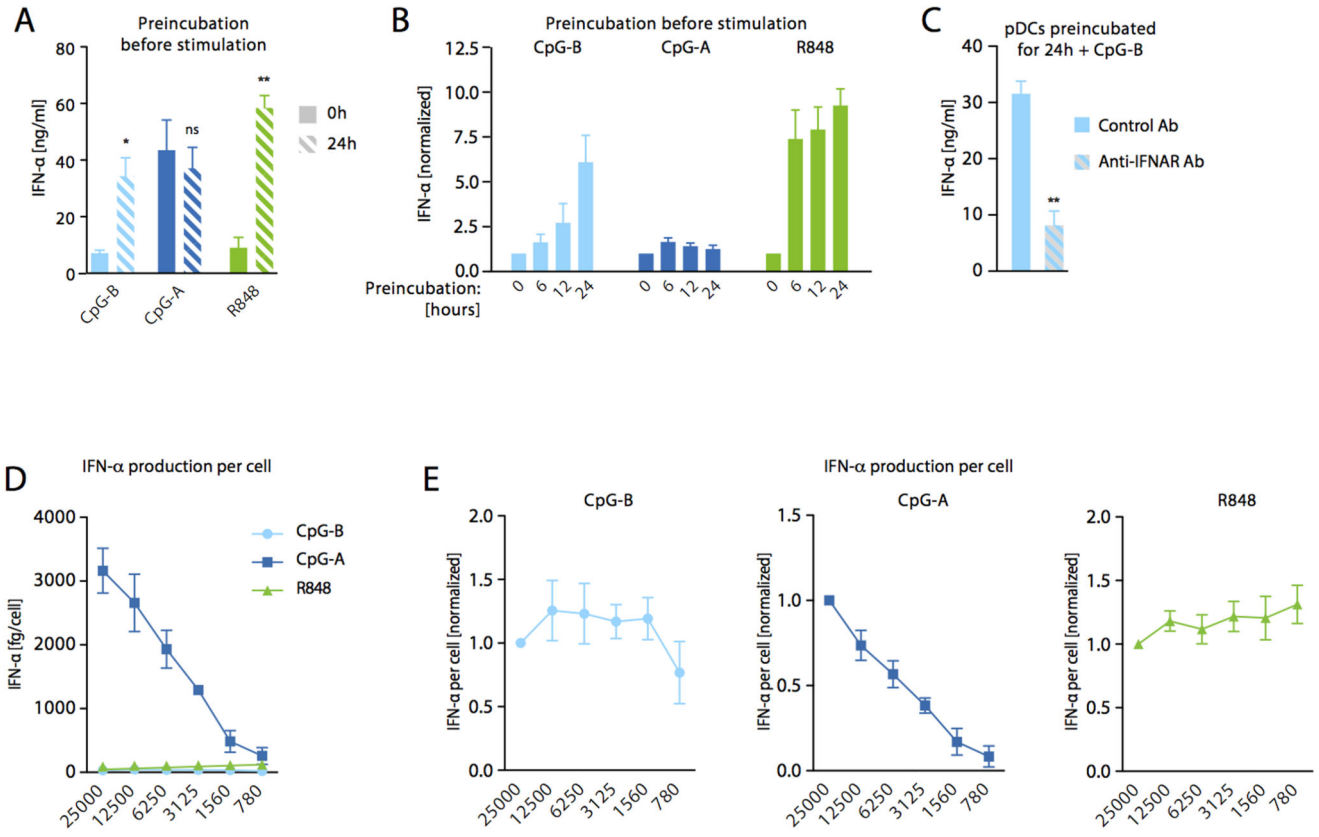


**Figure 4.**

Preincubation of unstimulated pDCs strongly primes toward higher IFN-α production. (A) pDCs were stimulated with CpG-A, CpG-B, or R848 either directly or after 24 h in vitro culture. Supernatants were collected 36 h after stimulation and IFN-α was measured by ELISA. (B) pDCs were preincubated in medium for the indicated periods and subsequently stimulated with CpG-A, CpG-B, or R848. Supernatants were collected 36 h after stimulation and IFN-α was measured by ELISA. Data shown are normalized to the reference condition (0 h preincubation). (C) pDCs were preincubated for 24 h prior to CpG-B treatment in the presence or absence of a blocking antibody against the type I IFN receptor. Supernatants were collected 36 h after stimulation and IFN-α was measured by ELISA. (D) pDCs were plated in 96-well plates in decreasing concentrations starting at 25 000 cells/well with serial dilutions (1:1) down to 780 cells/well. pDCs were stimulated with CpG-A, CpG-B, or R848. Supernatants were collected 36 h after stimulation and IFN-α was measured by ELISA. IFN-α production per cell was calculated by dividing the measured IFN-α production by the absolute cell number. (E) Data from (D) were normalized to the reference condition (25 000 cells/well). Results for (A–E) are shown as mean ± SEM of three samples pooled from three independent experiments. *$p < 0.05$, **$p < 0.01$ (paired Student's t-test).




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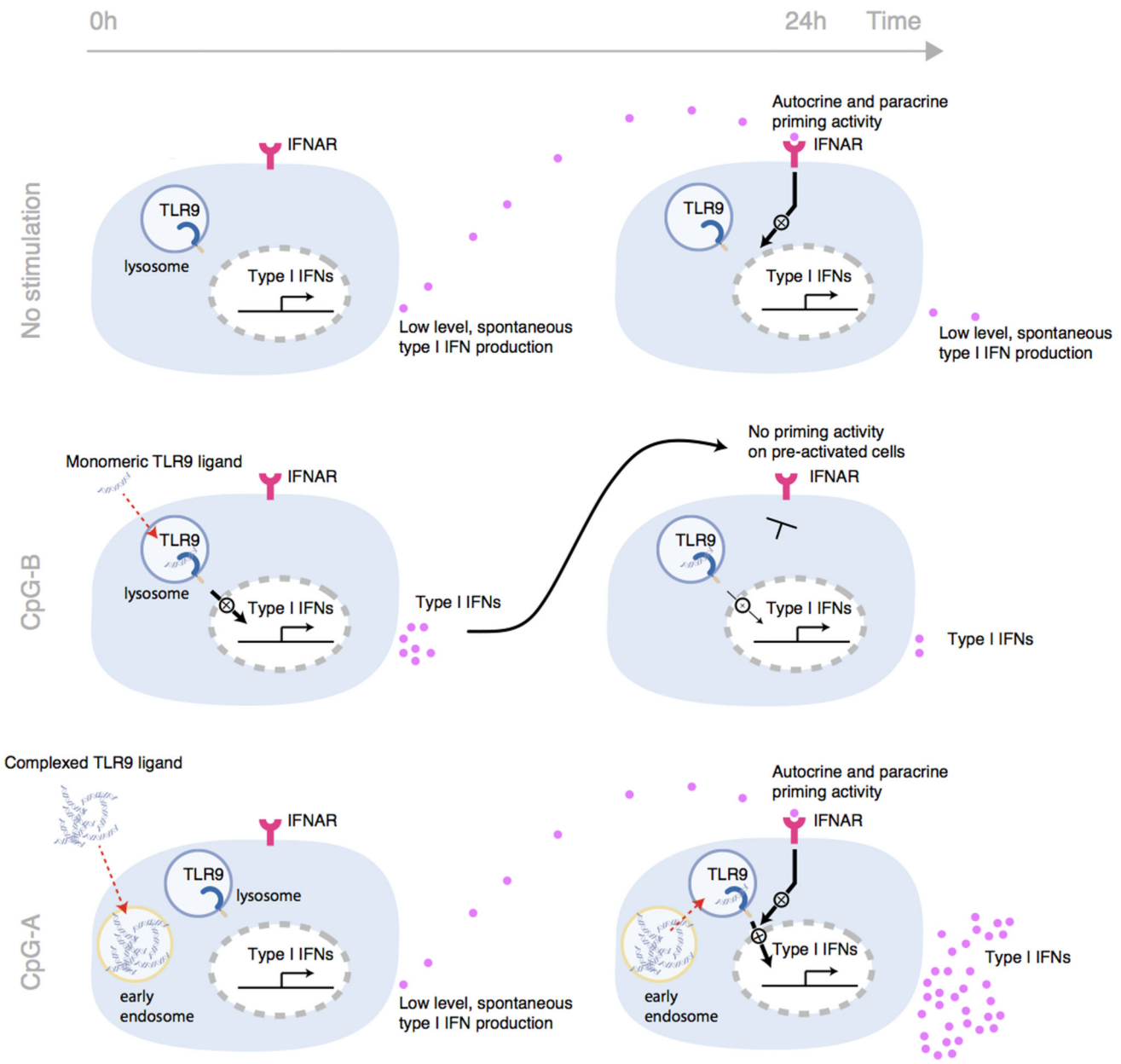


**Figure 5.**

Critical role of spontaneous, low level type I IFN production in self-priming of pDCs toward maximal type I IFN production. pDCs constitutively produce low amounts of type I IFN, which results in positive feedback regulation of their TLR-triggered type I IFN response. Monomeric CpG-B initiates a rapid activation of TLR9 signaling, with type I IFN production seen early as 2–4 h upon stimulation. Once activated via TLR9, pDCs cannot be preprimed by their auto/paracrine feedback loop and thus type I IFN production is not amplified. On the other hand, self-aggregating CpG-A triggers pDC activation in a delayed

fashion when pDCs have been primed by their spontaneous type I IFN feedback loop, resulting in a strong type I IFN response.



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