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Signalling pathways leading to IFN-α production in human plasmacytoid dendritic cell and the possible use of agonists or antagonists of TLR7 and TLR9 in clinical indications

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Abstract. Guiducci C, Coffman RL, Barrat FJ (Dynavax Technologies Corporation, Berkeley, CA, USA). Signalling pathways leading to IFN-α production in human PDC and the possible use of agonists or antagonists of TLR7 and TLR9 in clinical indications (Review). *J Intern Med* 2008; **265**: 43–57.

Plasmacytoid dendritic cells (PDC) are highly specialized immune cells capable of producing large amounts of type I and III IFN in response to viral infection. This response is mediated through TLR7 and TLR9 signalling pathways. In addition, PDC can differentiate into fully mature dendritic cells able to efficiently crosspresent viral antigens, thus playing an important role in adaptive immunity. This dual property of PDC is being used in clinical settings where synthetic TLR7 and TLR9 ligands are currently evaluated in clinical trials for the treatment of viral infections, allergies and cancers. Interestingly, there is evidence suggesting that chronic activation of PDC by endogenous RNA and DNA containing immune complexes maybe an important mechanism of driving autoimmunity and significant efforts to develop bi-functional antagonists of TLR7 and TLR9 are currently underway.

Keywords: autoimmunity, innate immunity, TLR7, TLR9.

Introduction

Plasmacytoid dendritic cells (PDC) represent a distinct DC population with a characteristic plasmacytoid morphology and abundant Endoplasmic reticulum (ER). In humans, PDC express a rather specific set of cell surface markers such as BDCA-4 and BDCA-2, as well as, very high expression of CD123 (IL3Rα). PDC also express CD4 whilst these cells do not express myeloid markers such as CD11c, which characterize conventional DC in human blood. In rodent, the pattern of markers is quite different as mouse PDC can be identified by CD11c together with B220, GR1, 120G8 and PDCA1 markers. In both human and mouse, PDC express a specific patterns of Toll-like receptor (TLR) being preferentially the nucleic acid specific TLR7 and TLR9 [1]. PDC represent a unique cell type as these cells have a major role in both innate immunity through the production of an impressive amount of type I and III IFN and adaptive immunity by their ability to mature into potent antigen presenting cells. The maturation process triggers dramatic morphological changes associated with increased expression of various co-stimulatory molecules that favours T cell activation [2–4].

In this article, we will review the key signalling pathways leading to IFN-α production in PDC and the possible applications of TLR7 and TLR9 agonists in the clinic. In addition, we will discuss the mechanisms by which chronic PDC activation by self

ligands can trigger autoimmunity and the possible use of antagonists of TLR7 and TLR9 as novel therapeutic agents for autoimmune diseases.

PDC role in linking innate and adaptive immunity immune response

The production of type I IFN by PDC can have a direct antiviral activity combined with a variety of indirect biological effects such as, increasing T cells survival and cytotoxic T lymphocyte (CTL) differentiation, Th1 differentiation, natural killer (NK) cell activity and upregulation of costimulatory molecules on conventional DC (cDC), overall boosting antiviral T-cell activity. IFN-α also induces phenotypical and functional maturation of blood monocytes into fully activated antigen presenting dendritic cells further contributing to antiviral immunity [5]. In addition, type I IFN can enhance antibodies response to soluble antigens and promote immunoglobulin class switch and immunological memory as well as induce the differentiation of B cells in plasmablast and subsequently into plasma cells [6, 7]. In contrast to cDC, IFN-α has a positive effect on PDC and is essential to their survival, activation and migration *in vivo* [8].

In addition, PDC have a key role in adaptive immunity and can activate CD4 and CD8 T cells. The nature of the stimuli has a dramatic effect on how PDC regulate CD4 response. Virus-stimulated PDC prime CD4 T cells to produce IFN-γ and IL10 but when activated with CD40L/IL-3, PDC promote Th2 differentiation and IL-4, IL-5 and IL-10 secreting CD4 T cells [9]. In contrast, some reports have suggested that both human and mouse PDC have the ability to prime naïve CD4 T cells to differentiate in IL-10 producing regulatory T cells, possibly contributing to the maintenance of tolerance [10, 11].

Until recently the ability of PDC to crosspresent antigens to CD8 T cells was subject to many controversies. Indeed, murine PDC can crosspresent antigens only in specific settings that involve concomitant TLR triggering with CpG-containing immunostimulatory sequences (CpG-ISS) and relative high frequencies of T cell precursors whilst in human no clear evidence of the ability of PDC to crosspresent antigens are available, with most of the studies performed in mixed lymphocyte reaction (MLR) settings [12]. Two recent reports, however, showed that human PDC are indeed highly capable and efficient in crosspresenting viral antigens. Hoffel *et al.* showed that human blood PDC can crosspresent HIV-derived peptides to CD8 HIV-specific T cells under the form of lipopeptide-coniugates or HIV-infected cells undergoing apoptosis [13]. The ability of stimulating differentiation of CD8 T cells did not appear to be dependent on TLR triggering and IFN production, as it was observed in naïve/unstimulated PDC or IL-3 treated PDC. However, the process was greatly enhanced by influenza virus, suggesting that, *in vivo*, co-triggering of TLR by a virus could boost the ability of these cells to present viral antigens to CD8 T cells [13]. More recently, Di Pucchio *et al.* provided the mechanism of crosspresentation in human PDC [14]. The authors showed that upon viral encounter, human PDC but not cDC undergo a radical transformation becoming highly efficient in crosspresenting viral antigens. Keys to this phenomena are: (i) their ability to load antigens on MHC class I directly in the early recycling endosomal compartment with no need of transport in the cytoplasm and (ii) the rapid targeting of class I molecules into the early endosome, resulting in cross-presentation measurable only after 4 h. PDC can mount a fast response to viral recognition by producing high level of type I IFN and inducing class I presentation which may likely be essential in controlling early viral replication [14]. Accordingly, in mice, depletion of PDC was found to impair cytotoxic T lymphocytes (CTL) generation to cutaneous herpes simplex virus (HSV) [15], respiratory syncytial virus (RSV) clearance in the lung [16] and HSV-2 viral clearance and IFN-α production in the genital mucosa [17].

Signalling pathways leading to IFN-α production in PDC

Human PDC recognition of pathogens is mediated primarily through the recognition by TLR7 and TLR9 of the nucleic acids of the invading organism, RNA and DNA respectively. PDC produce type I IFN in

response to a wide variety of RNA and DNA viruses such as HSV, Sendai, HIV-1, influenza, Newcastle, vesicular stomatitis virus (VSV) and in response to bacteria (SAC) and parasites (*Plasmodium falciparum*). In contrast to most cell types, PDC have an almost exclusive TLR-dependent ability to produce type I IFN. Other nucleic acid cytosolic receptors of the helicase family such as RIG-I or MDA5 have limited to no role in IFN-α induction from PDC [18]. Synthetic TLR9 and TLR7 ligands such as CpG-ISS and imidazoquinoline compounds are widely used and trigger IFN-α production as well [2, 19]. The IFN response by PDC is remarkable with over 50% of the induced RNA transcripts following TLR7 or TLR9 triggering encoding for type I IFN, resulting in a production of about 3–10 pg/cells of IFN-α protein at 24 h, 100–1000 more then any other cell type in the blood [3]. Interestingly, other cell types such as B cells which express TLR7 and TLR9 are unable to produce IFN-α upon activation, underlying the peculiarity of PDC in this regard. The pathway leading to IFN-α production depends on IRF-7 activation and its migration to the nucleus [20]. Even though many signalling molecules are important for the IFN pathway, they all rely on their ability to activate IRF-7. IRF-7 forms a complex with MyD88, IRAK-1, IRAK-4 and TRAF6, with IRAK-1 directly phosphorylating IRF-7 [2, 19]. TLR7 and TLR9 activation not only induce IFN-α in PDC but also proinflammatory cytokines such as TNF-α and IL-6 and upregulation of costimulatory molecules [3]. The initiation of the signalling cascade requires the activation of a signalling complex constituted by MyD88, IRAK-4 and TRAF6. Downstream to this complex the resulting response bifurcates with a branch leading to nuclear factor kappa B (NF-κB) activation and the other to the activation and translocation of IRF-7 (Fig. 1). Mice deficient for MyD88, IRAK-4 or TRAF6 have defects in both the NF-κB and IRF-7 pathway, suggesting that these are upstream of the more specialized signalling

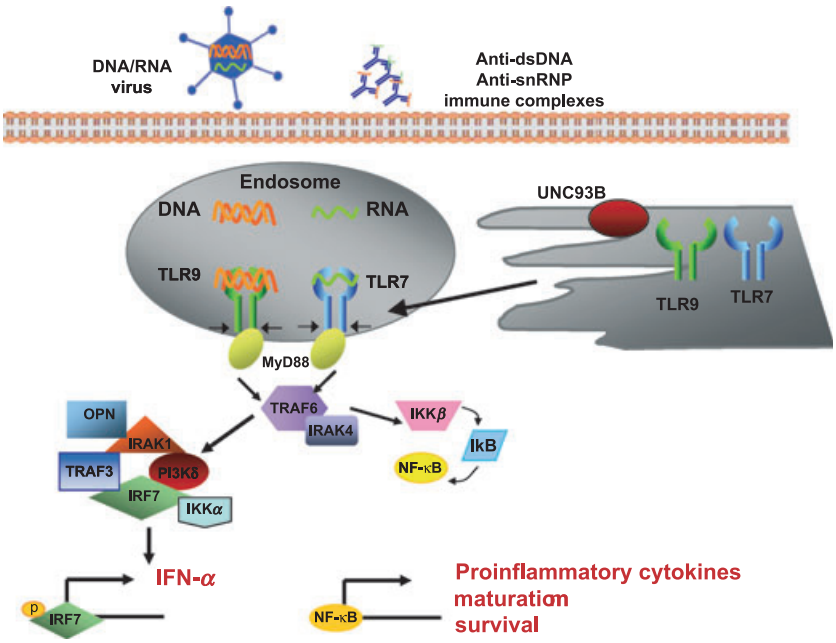

Fig. 1 Toll-like receptor (TLR)7 and TLR9 signalling pathway in plasmacytoid dendritic cells (PDC). In PDC, TLR7 and TLR9 localize in the endoplasmic reticulum in unstimulated cells. Upon stimulation with TLR7 and TLR9 ligands, chaperon protein UNC93B mediates transport of the receptors in the endosome where the binding with RNA and DNA molecules induce a conformation change and formation of the signalling complex, which includes MYD88, TRAF6 IRAK4. Downstream of this complex, the resulting response appears to be bifurcated: the nuclear factor kappa B (NF-κB) pathway gets activated and leads to the induction of proinflammatory cytokines and the acquisition of antigen presenting functions. The IRF7 pathway leads to Type I, III IFN production and depends on numerous coactivators including IRAK1, TRAF3, OPN, IKKα and PI3Kδ.

molecules [21–25]. The pathway that leads to NF-κB activation is postulated to be the classical one involving the activation of the IKK complex (IKKβ,γ) which, by catalysing the phosphorylation of IkBα protein and its degradation permits NF-κB to access the nucleus [24, 26]. Mouse PDC deficient for IRAK-1 have an intact NF-kB response but are unable to produce IFN-α in response to both TLR7 and TLR9 ligands [27]. In a similar manner, IKK-α deficient PDC show a selective defect in the IRF-7 branch of the TLR7 and TLR9 response [28]. Both proteins bind to IRF-7 and are necessary for its migration in the nucleus. More recently, other adaptors have been found to be essential for IFN production, strongly suggesting a tight regulation of this branch of the PDC response to pathogens. Indeed, OPN and TRAF3 deficient mouse PDC do not produce IFN-α in response to TLR7 and TLR9 ligation, although is currently not clear how they modulate IRF7 activation [24, 29, 30]. In addition, in human PDC, PI3Kdelta was recently demonstrated as necessary for nuclear translocation of IRF-7 and IFN-α production whilst having a limited role in the NF-κB pathway [31]. It remains to be elucidated how these molecules act together to activate IRF-7.

Mechanism regulating PDC response in human

As PDC can participate to both innate and adaptive immunity when activated through the same receptors, TLR7 and TLR9, this raises the question about what mechanisms are regulating this process. One of the more likely explanations is that PDC possess an extremely sophisticated mechanism to compartmentalize the response to TLR7 and TLR9 [32, 33]. In both human and mouse, PDC uncouple the two responses, IFN-α versus maturation/inflammatory cytokines, by properly directing TLR9 ligands in the early or late endosomal compartment respectively. Indeed, there is a consistent correlation between the nature of the PDC response to TLR9 ligation and the intracellular localization where the interaction between ligand and TLR9 primarily occurs [33]. This was demonstrated using fluorescent-labeled CpG-ISS of different classes that are known to induce either IFN-α (CpG-A) or maturation (CpG-B). CpG-A ISS form multimeric structures of the size of microparticles whilst CpG-B are single stranded oligonucleotides. The pattern of endosomal distribution between these two CpG-ISS is dramatically different in human and mouse PDC [32, 33]. CpG-A are preferentially retained in the early endosomes of the PDC and this correlates with the induction of high IFN-α levels and inefficient upregulation of costimulatory molecules expression. Conversely, single stranded CpG-ISS (CpG-B class) cannot activate PDC to produce IFN-α consistently with their localization in the late endosomal compartments [32, 33] (Fig. 2). Interestingly when the multimeric structure of CpG-A is destroyed and rendered single stranded, the oligo traffics to the late endosomal compartment and loses its ability to produce high IFN-α. Conversely, complexing CpG-B with the cationic peptide polymixin generate the formation of large multimeric structures where the oligo is embedded in the structure. This results in an increase in the retention time in the early endosome and induction of large amount of an IFN-α associated with loss of maturation induction (Fig. 2). When CpG-ISS are complexed in highly ordered structures, this may mimic viral DNA accumulating in the endosome. In addition, PDC might possess highly specialized mechanisms to control the intracellular trafficking of TLR9 ligands, as mouse conventional DC which also express TLR9 are unable to properly localize TLR9 ligands in the early endosome despite their large structures, failing to produce IFN-α [32].

The response following TLR7 or TLR9 triggering is also dependent on the trafficking of the receptors themselves from the ER to endosomes [34, 35]. This has been reported mostly using cell lines and it is unclear whether this is also the case in cells such as PDC. The transport of TLR7 and TLR9 to the endosome requires a chaperone protein named UNC93B, in absence of which PDC are unable to respond to TLR stimulation [35–37]. Patients deficient for this protein do not respond to TLR3, TLR7 and TLR9 ligation [38]. The challenge is now to elucidate how UNC93B sense DNA and RNA molecules to start the translocation of the receptors from the ER to the endosome.

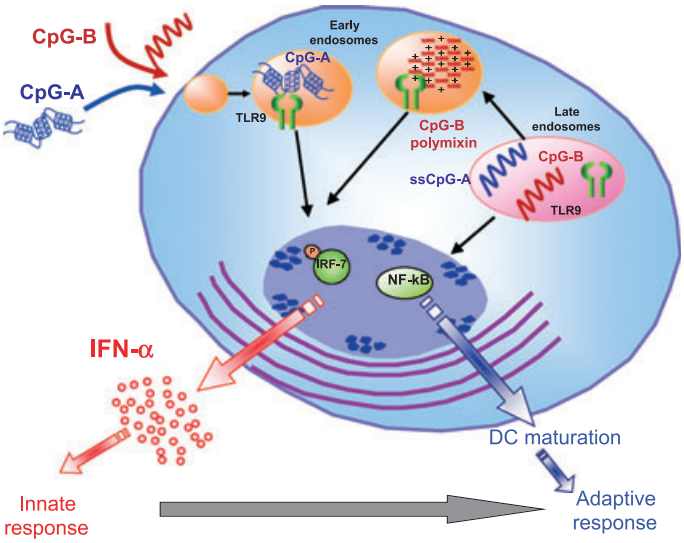

Fig. 2 Compartmentalization of Toll-like receptor (TLR)9 response in plasmacytoid dendritic cells (PDC). In PDC, CpG-A TLR9 ligand, which form large aggregate structures, is retained in the early endosomal compartment and induce abundant IFN-α through the IRF7 pathway. Conversely, single stranded CpG-B accumulates preferentially in the late endosome lead to the activation of the NF-kB pathway and induction of maturation, survival and proinflammatory cytokines production. This model is further supported by data showing that rendering CpG-B a multimeric structure by polymixin conjugation completely changes the biological outcome of TLR9 triggering leading to strong IFN-α production. Consistently, CpG-A rendered monomeric (single stranded) is found to the late endosome and loses most of its IFN-α induction ability.

Clinical development of TLR7 and TLR9 agonists

In the past years an increasing number of novel immunotherapeutic approaches have been developed based on activation of the innate response via TLR triggering. TLR7 and TLR9 agonists are currently being investigated in clinical trials to improve vaccine efficacy, in the treatment of allergies and in cancer therapy. The following section will review the rationale and future prospects of these approaches.

Synthetic oligonucleotides rich in CpG motifs named CpG-ISS trigger TLR9. CpG-ISS are typically synthesized with a phosphorothioate backbone (PS) to confer nuclease resistance and increase the half-life. Three different classes of CpG-ISS (Class A, B, C) have been described so far, these differ not only in sequence but also in their biological response as well (Table 1). In clinical trials, CpG-ISS of the class B have been the more extensively investigated. As TLR9 expression in human blood is mainly confined

Table 1 Characteristic of ISS classes

ISS Class	Structural characteristics	Activity on PDC	Activity on B cells
CpG-A	Forms higher-molecular weight aggregates through G-tetrads	High IFN-α Low maturation	Low
CpG-B	Single strand	Low IFN-α High maturation	High
CpG-C	5′-TGC with 12-nucleotide of palindromic sequence; forms duplexes	High IFN-α High maturation	High

Single stranded CpG-B induce moderate IFN-α production in plasmacytoid dendritic cells (PDC) and strong maturation with upregulation of costimulatory molecules, on the opposite, multimeric class A CpG-A is an excellent inducer of IFN-α in PDC whilst being a poor stimulator of maturation. CpG-B activates strongly B cells to proliferate, upregulate costimulatory molecules and produce IL-6, whilst CpG-A has very limited B cell stimulatory activity. Double stranded class C CpG-C combines the properties of Class A and B being a strong inducer of IFN-α and maturation of PDC and strong activator of B cells.

Table 2 Clinical development: vaccine adjuvants, asthma and cancer

Indication	Compound	Target	Company/institution	Status
Prophylactic infection disease				
Hepatitis B	Heplisav™ (HBV surface antigen and CpG-B 1018 ISS)	TLR9	Dynavax Technologies	Phase III (ongoing; ref. [44])
Anthrax	VaxImmune™ (CpG7909, a CpG B class ODN) with approved anthrax vaccine (BioThrax®)	TLR9	Coley Pharmaceutical/Pfizer	Phase I (completed; ref. 45, 46)
Influenza	Influenza antigens and CpG-ISS	TLR9	Dynavax Technologies	Preclinical
Malaria	AMA1-C1/Alhydrogel®+ CpG7909	TLR9	NIAID	Phase (completed; ref. [47])
Therapeutic infection disease				
Papilloma-induced genital warts	Aldara™ (imiquimod cream 5%)	TLR7	3M Pharma	Approved
Hepatitis C	IMO-2125 (CpG-ISS)	TLR9	Idera	Phase I (ongoing)
Hepatitis C	CpG-10101 (CpG-C)	TLR9	Coley Pharmaceutical/Pfizer	Phase I suspended
Hepatitis C	ANA733	TLR7	Anadys	Phase I (ongoing)
Hepatitis C	CpG B and CpG C class ODN	TLR9	Dynavax Technologies	Preclinical
Cancer				
Basal cell carcinoma	Aldara™ (imiquimod cream 5%)	TLR7	3M Pharma	Approved
Nonsmall-cell lung cancer	MAGE-3A plus CpG-B (CpG7909)	TLR9	GlaxoSmithKline	Phase III (ongoing)
NonHodgkin's lymphoma	1018 ISS (CpG B class ODN plus Rituxan)	TLR9	Dynavax Technologies	Phase II (completed)
Metastatic Colorectal cancer	1018 ISS (CpG B class ODN in combination with chemotherapy)	TLR9	Dynavax Technologies	Phase I (completed)
Nonsmall-cell lung cancer	IMOxine® (HYB2055, IMO-2055; CpG-ISS) plus chemotherapy Tarceva® and Avastin®	TLR9	Idera Pharmaceuticals	Phase I (ongoing)
Renal cell carcinoma	IMOxine® (HYB2055, IMO-2055; CpG-ISS)	TLR9	Idera Pharmaceuticals	Phase II (ongoing)
Melanoma	CYT004-MelQbG10 (Melan-A/MART-1 protein fragment coupled to immunodrug carrier QbG10 CpG-ISS)	TLR9	Cytos Biotechnology	Phase IIa (ongoing)
Allergic Disease				
Allergic rhinitis (ragweed)	Tolamba™ (Amb a 1 ragweed allergen with covalently linked CpG B class ODN 1018 ISS)[62]	TLR9	Dynavax Technologies	Phase II/III (completed)
Allergic rhinitis (dust mite)	CYT005-AllQbG10 (allergen extract of house dust mite mixed with immunodrug carrier QbG10)	TLR9	Cytos Biotechnology	Phase II (completed)
Asthma	Second generation CpG-ISS	TLR9	Dynavax Technologies/AstraZeneca	Preclinical
Asthma	HYB2093 (CpG-ISS)	TLR9	Idera Pharmaceuticals/Novartis	Preclinical

Additional references or press releases can be found on companies websites (http://www.gsk.com, http://www.dynavax.com, http://www.coleypharma.com, http://anadyspharma.com,// www.cytos.com, http://www.iderapharma.com). NIAID, National Institute of Allergy and Infectious Diseases; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; ISS, Immunostimulatory sequence.

to PDC and B cells, these two subsets are believed to be the main responders and contributors to the therapeutic effect of CpG-ISS following *in vivo* administration. CpG-ISS are believed to activate the immune system by the direct induction of IFN-α which, as already discussed, is a strong Th1 skewing cytokine. In addition, by activating B cell proliferation, antibody production and isotype switching, CpG-ISS can boost the humoral branch of the adaptive response [39]. Small molecules triggering TLR7 such as imidazoquinoline-based compounds (Imiquimod, Gardiquimod, Resiquimod) or guanosine analogues (Loxoribine) are being investigated as well, however, most of the data obtained in clinical studies have been obtained with Imiquimod. Similar to ISS, TLR7 agonists directly activate innate immune effectors cells being potent inducer of IFN-α and proinflammatory cytokines (TNF-α, IL1α/β, IL-6, IL-8) *in vivo* [40].

TLR7 and 9 agonists in infectious disease

For the prevention of infectious diseases, the TLR9 ligands ISS have been extensively investigated as vaccine adjuvant for prevention of hepatitis B virus (HBV) influenza, malaria and anthrax [39, 41–43] (Table 1). An HBV vaccine, currently in Phase III clinical trails, combining CpG-1018 ISS with Hepatitis B surface antigen has proven able to overcome the age-related decline in responsiveness observed with conventional HBV vaccine [44]. In Phase I/II clinical trials, CpG-B ISS (CpG-7909) combined with an approved HBV vaccine, Engerix-B, significantly improved antigen-specific antibody response in healthy individuals and in previously non responsive HIV-infected individuals [45, 46]. Another trial that used CpG-B ISS combined to an approved anthrax vaccine showed a higher and earlier antibody response [41]. In addition, CpG-B combined to a poorly effective malaria vaccine (AMA1-C1) was reported to strongly increase antibody response, which was demonstrated to be functionally active in preventing parasite growth *in vitro* [47]. CpG-B was also tested in a Phase Ib as an adjuvant of a commercial influenza vaccine. A lower dose of the vaccine substantially decreased antigen specific IFN-γ response, the addition of CpG-B restored the response suggesting that it could be possible to use ISS to reduce the dose of vaccine without compromising the efficacy [48]. These early clinical data confirm the preclinical data obtained in mouse demonstrating an increase and more sustained immunogenicity of pre-existing vaccines when combined with ISS molecules.

The ability of TLR7 and TLR9 agonists to elicit protective immunity during viral infection has been largely explored in mice models and in few human clinical trials. The high amount of type I IFN-α induced by TLR7 and TLR9 agonists is believed to account for their antiviral activity; in addition the indirect effect of Type I IFN-α on other cells of the immune system such as CTL and NK could further contribute to their mechanism of action. TLR7 agonist, Imiquimod (5% cream) is the most advanced in this context, with an already approved indication for Papilloma induced genital warts [49]. Cumulative results from five clinical trials on immunocompetent patients reported a complete clearance of the virus in 51% of the treated patients [50]. Other infectious diseases of the skin have been reported to respond to Imiquimod treatment including molluscum montagiosum, genital herpes and leishmaniasis [43]. A small molecule-based TLR7 agonist, given orally, has been explored in HCV infected patients; although clinical data were promising, the product development was halted by a study in animals showing toxicity when the drug was given on a daily basis [51]. After additional preclinical studies the clinical investigation of this compound in HCV infected people has been resumed. Therapeutic activity of a CpG-C ISS was investigated in patients affected by chronic hepatitis C where it was found to induce a dose-dependent reduction of viral RNA in the blood. High level of IFN-α, IFN-α inducible-chemokines as well as increased NK cell activity was reported [52]. Despite these promising results, no long-term clinical efficacy was observed and the program was suspended [53].

TLR7 and TLR9 agonists in allergies

Allergy is another field where TLR9 agonists have seen a significant clinical development. Allergic

diseases are characterized by an inappropriate Th2 T cell response to environmental antigens. TLR9 agonist ISS are particularly suited for the treatment of allergic disease as they can promote Th1-response *in vivo*, which is typically beneficial in allergic response. Numerous preclinical mouse models have shown that the ability of ISS to induce IFN-α and proinflammatory cytokines such as IL-12 contribute to its therapeutic activity. Preclinical studies in monkey asthma models demonstrate that ISS are capable of inhibiting airways hyper-responsiveness and airway remodelling; these preclinical studies are particularly relevant as the cellular distribution of TLR9 in monkeys, unlike mice, resemble that in man [39]. In human clinical trials, ISS covalently linked to the allergen Amb a1, administered to ragweed allergic patients, reduced hay fever symptoms significantly when compared with placebo recipients [54, 55]. A Phase II clinical trial in patients suffering from dust and cat allergies with CpG-ISS containing immunodrug (CYT003-QbG10), showed significant efficacy compared with placebo in reducing rhinoconjunctivitis symptoms.

TLR7 and TLR9 agonists in cancer

The field in which TLR9 and TLR7 agonists has been most extensively investigated is cancer therapy. The rationale behind the use of TLR7 or TLR9 agonists relies on the properties of these compounds to (i) induce IFN-α and proinflammatory cytokines which by inducing DC maturation into fully competent antigen presenting cells that would facilitate the mounting of an effective CD8 T cell response against tumour antigens; (ii) overcome tolerance against tumour antigens which are typically poorly immunogenic; (iii) overcome the highly inhibitory environment created locally by the tumour itself by inducing a potent innate response in the local environment.

TLR7 agonists are perhaps the most promising form of immune agents capable to induce tumour regression. Imiquimod based cream is already approved for the treatment of basal cell carcinoma. In a multicenter Phase III study, tumour clearance was demonstrated in 80% and 6% of subjects treated with Imiquimod and vehicle cream respectively [56]. Imiquimod was shown to induce a marked increased of gene expression involved in the innate response. In particular, PDC seems to have a direct functional role in its clinical efficacy. Indeed, gene expression profiling of treated basal cell carcinoma tumours have shown to induce a specific IFN-α gene signature that strictly correlate with the presence and abundance of PDC infiltrating the tumour lesions; patients who did not mount a clinical response to the treatment were shown to have less PDC infiltration and lower IFN-α signature [57]. In addition to basal cell carcinoma, Imiquimod has been demonstrated to be extremely effective against a variety of primary skin tumours including kerathoacantomas, actin keratosis, primary melanoma *in situ* and cutaneous T cells lymphomas [40].

Although no drugs containing TLR9 agonists have already been approved for cancer therapy, some encouraging results were obtained in clinical trials. Recently, a Phase II trial on patients affected by non-small-cell-lung carcinoma (NSCLC) and treated with MAGE-A3 formulated with CpG-B-ISS, showed a 27% reduction in the relative risk of recurrence following surgery compared with surgery only. These promising results have led to an on going Phase III clinical trial.

Combination of CpG-B with Melan-A/MART-1 was tested in metastatic melanoma patients. The expansion of Melan-A specific CD8 T cell response was 10 fold higher in patients immunized as compared with patients receiving the same antigen but not ISS [58]. Circulating CD8 T cells were characterized to be fully competent cytolitic T cells. However, this immunological achievement was not sufficient to observe a regression of the disease. More in depth analysis of the reasons for clinical failure demonstrated that tumour specific infiltrating CD8 T cells obtained from patients' biopsies differ in their functional capacity as compared with their blood counterpart, showing lower level of cytotoxic factors such as perforin and granzyme A/B and low secretion of IFN-γ. The presence of poorly functioning Melan-A T cells was accompanied by the presence, in the tumour, of numerous FOXP3 regulatory T cells [59]. These observations correlate well with both preclinical mouse studies and

other clinical data showing that the tumour microenvironment favours immune tolerance by promoting expansion of regulatory T cells, by rendering dendritic cells unable to leave the tumour and migrate to the site of antigen presentation and by paralysing infiltrating innate effectors cells such as macrophages [60, 61]. Clinical data accumulated, so far, suggest that the optimal strategy will be to combine ISS with multiple therapies such as surgery, radiation, monoclonal antibodies and cytotoxic drugs.

Role of PDC in autoimmune disease

The intracellular-endosomal localization of TLR7 and TLR9 in PDC is believed to be the key for three aspects of their biology: (i) concentration of nucleic acid in specialized endosomes, so that the acidic environment allows the degradation of the pathogens and release of the nucleic acid; (ii) defines the nature of the response and thus the role of PDC in innate or adaptive immune response; (iii) protection against recognition of self DNA or RNA in the extracellular environment limiting unwanted autoimmune response [62]. Indeed, TLR9 can recognize both pathogenic and self DNA. When using an engineered version of TLR9 that forces expression of the receptor on the cell surface, then TLR9 can respond to CpG-ISS and self DNA whilst activation by viruses is decreased [63].

Autoimmune diseases develop when the adaptive immune response target host tissues. A large body of evidence suggests that in certain autoimmune diseases, the deregulation is not exclusively in the adaptive branch of the immune response but in the innate one as well. A deregulated innate response by boosting antigen presentation and suppressing regulatory T cells activity can results in an overacting adaptive response against self antigens.

Systemic lupus Erythematosus (SLE), is an excellent example in this regard; an autoimmune disease where the innate tolerance to self nucleic acids is broken with devastating consequences. SLE is a relapsing, remitting disease for which no new therapy has been approved in the past 30 years [64–66]. Patients suffer from kidney dysfunction, leading to renal failure and a wide and variable range of symptoms, including arthritis, fever, skin rashes and brain inflammation. An increasing number of confirmed loci have been reported to contribute to SLE (i) inability to clear immune complexes due to decreased copy of FCγRIII [67]; (ii) inability to efficiently clear DNA released from dying cells due to mutation in the DNase 1 [68]; (iii) polymorphism in IFN regulatory factor 5 (IRF5) [69]; (iv) polymorphism in PTPN22[70], STAT4 [71]; (v) decreased level of BLK [72].

The hallmark of the disease in an increased IFN-α signature in the blood and in skin which is tightly associated with increased detectable levels of autoantibodies and disease activities [73–75]. The concept that increased IFN-α levels plays a direct role in the disease is supported by at least four observations: (i) clinical data shows nonautoimmune patients developing lupus like syndrome and accumulation of autoantibodies if treated with soluble IFN-α [76, 77]; (ii) viral infections, UV skin injury or other events leading to IFN-α induction are known to be activators of lupus flares; (iii) lupus patients undergoing disease flares and treated with high dose corticosteroid (CS) show a decrease number of circulating PDC accompanied by prompt decrease of the IFN signature [74]; (iv) NZB mice, which spontaneously develop a lupus-like disease, have less severe disease with delayed onset when made deficient for the IFN-α receptor [78]. Moreover, lupus patients exhibit elevated levels of neutrophil-specific genes such defensins and granulopoiesis-related genes [74]. It is interesting to note that both self DNA/chromatin and snRNPs containing RNA are increased in SLE consistent with a role for both TLR7 and TLR9 in the disease. Many studies showed that PDC are the source of IFN-α when human PBMC are activated by anti-DNA and anti-RNP immune complexes [79–81]. This process requires the expression of FcγRIIa on the surface of PDC, allowing efficient delivery of the nucleic acid-containing antibodies complexes into the TLR7 and TLR9 containing endosomal compartments [80, 82].

There are interesting hypotheses on how the deregulation of IFN-α and RNA/DNA autoantibodies

production contributes to lupus pathogenesis. (i) IFN-α is a strong promoter of B cell differentiation into antibody producing plasmocytes [7] and can induce B cell survival factors such as BAFF [83]. This leads potentially to a positive feedback loop in which antibodies produced by autoreactive B cells can activate IFN-α from PDC which in turn promotes B cell survival, activation and differentiation; (ii) IFN-α stimulate and maintain the differentiation of blood monocytes into fully activated antigen presenting cells which could in turn activate autoreactive T cells that have escaped central tolerance [5, 84]; (iii) DNA and RNA containing IC are believed to further boost the production of autoantibodies in a process that requires simultaneous engagement of the BCR and TLR7 or TLR9 receptors on the B cells [85–87]; (iv) IFN-α has been shown to activate neutrophils towards activation and production of reactive oxygen intermediates (ROI) which mediates the end-organs damage [88] (Fig. 3).

Interestingly, Type I IFN and PDC have been proposed to contribute to the pathogenesis of other autoimmune diseases accompanied by IFN-α signature. Conspicuous number of PDC accumulate in the pancreas, muscle and salivary glands of people affected by diabetes mellitus, dermatomyositis and Sjorden's syndrome, strongly suggesting that deregulated PDC response to self could be a more general phenomenon in driving autoimmune diseases [66, 89] (Table 1). Of particular interest is the link between IFN-α and cutaneous skin diseases characterized by interface dermatitis (ID) [90]. The term ID refers to a specific histological inflammatory pattern that is characterized by vacuolar changes (liquefaction), apoptotic keratinocytes, accumulation of cytotoxic CD8 T cells

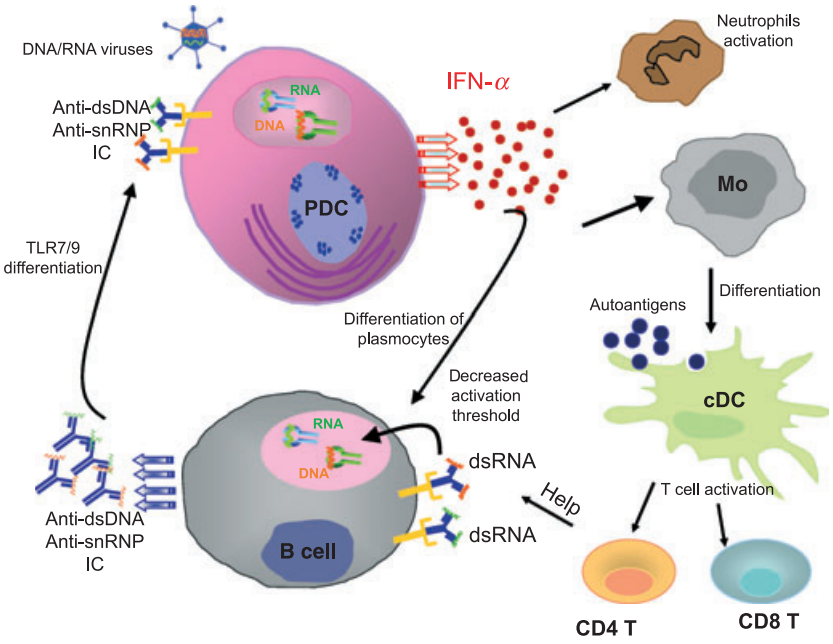

Fig. 3 Proposed role of a deregulated innate response to self Toll-like receptor (TLR)7 and TLR9 ligands in lupus. Nucleic acid-containing immune complexes can activate plasmacytoid dendritic cells (PDC) and induce IFN-α synthesis, which can further enhance autoantibody production by B cells specific for double stranded DNA (dsDNA) and ribonucleoproteins (snRNP) by stimulating the differentiation of naïve B cells in plasmocytes. Co-triggering of BCR and TLR7 and TLR9 in B cells lower the threshold of activation needed for the production of autoantibodies, further increasing the level of self-ligands able to trigger IFN-α response in PDC. IFN-α itself stimulates the differentiation of blood monocytes in fully activated antigen presenting cells able to present autoantigens to self reactive CD8 and CD4 T cells. In addition IFN-α leads to activation of neutrophils which can even respond to IC; dying neutrophils could constitute an additional source of autoantigens which cDC can uptake and process.

and IFN-signature in the skin [90]. Diseases such as lichen planus, dermatomyositis, lichen sclerosus, cutaneous lupus and cutaneous GVHD fall under this category [90]. The close association between IFN-α-producing PDC and granzyme B positive T cells together with the observed accumulation of IC at the junction of dermis and epidermis [91] suggest that IFN-α has a major role in fueling cellular immune response [90, 92, 93]. Another very interesting skin autoimmune disease which shows a clear PDC infiltrate linked to IFN-α signature is psoriasis. Psoriasis has a different histological characteristic than ID and no IC are found deposited in the skin but recently, an accumulation of an anti-microbial cationic peptide, LL-37, was reported [94]. LL37 was shown to shuttle self DNA in the early endosomal compartment of PDC, stimulating TLR9 and subsequent production of IFN-α possibly explaining the fact that PDC infiltrating psoriatic lesions produce IFN-α [94]. Blocking IFN-α or PDC ability to produce IFN-α, in a xeno-transplant model of human psoriasis prevented the development of the disease [95]. As anti-microbial peptides can be induced following tissue injury, this suggest that (i) similar pathways might be involved in other inflammatory situations and (ii) that the optimal therapeutic approach to block this activation signal would be at the TLR level and not by targeting each individual peptide [66].

Possible therapeutic use of TLR7 and TLR9 antagonist in autoimmune diseases

Based on the rationale presented above, inhibiting the IFN-α pathway offers promise for autoimmune disease such as SLE and related diseases involving the same pathway. Clinical data stands behind this rationale; therapeutic benefits are observed in SLE patients treated with chloroquine or glucocorticoids that inhibits IFN-α production [96] or down regulate IFN-α signature by killing PDC [74, 97]. However, both chloroquine-like agents and glucocorticoids have intolerable side effects.

Blocking antibodies to IFN-α are currently being tested in Phase I/II clinical trials. The results of a short Phase I trial showed a dose-dependent reduction of IFN-signature in the blood with no apparent side effects [97].

Another approach to target IFN-α pathway is to use inhibitors of both TLR7 and TLR9. In this regard, we and other have described several distinct subsets of nonstimulatory DNA sequences that can inhibit TLR7 and TLR9 stimulation both *in vitro* and *in vivo* [66, 79–81]. We have named this novel class of sequences IRS (ImmunoRegulatory Sequences) and have shown that they block PDC production of type I IFN not only by synthetic ligands (R-848 for TLR7 and CpG-ISS for TLR9) but also by the presumed natural ligands for these receptors such as RNA, DNA viruses and anti-DNA and anti-RNP IC from lupus patients [81]. Similarly, IRS are able to block TLR7 and TLR9 mediated proliferation and cytokines production by human B cells [98]. In addition, lupus-prone mice treated with IRS showed reduced levels of autoantibodies, proteinuria and glomerulonephritis associated with increased survival [99]. These new inhibitors are predicted to have therapeutic benefit for lupus by: (i) inhibiting PDC, the major source of IFN-α contributing to the pathogenesis of lupus, without blocking low levels of IFN-α and IFN-α induced by several other pathways in other cell types; (ii) inhibiting activation of anti-DNA and anti-RNP-specific B cells and consequent production of anti-nucleic acid autoantibodies; (iii) possibly lowering the activation status of neutrophils, decreasing tissue damage. There are two possible ways by which these inhibitors blocks TLR7 and TLR9 signalling, by competing with the ligands for binding to the receptors or by inhibiting a signalling component downstream of both receptors. Although it is difficult to exclude the second hypothesis, a recent paper on the mechanism of signal transduction by TLR9 [100] strongly suggest this inhibitors acts by antagonizing the receptor. This elegant study showed that TLR9 exists in intracellular membranes as a pre-formed homodimer. When a stimulatory ODN binds to the ligand-binding domains of this dimer, it causes a conformational change that brings their cytoplasmic domains closer together, resulting in signal transduction. The authors used a TLR9 inhibitory ODN and shown

that although it binds to the receptor as efficiently as the stimulatory ODN it was unable to induce the conformational change.

One of the major concerns of targeting the IFN-α pathway is that when used chronically, as one would expect in autoimmune disease settings, it could lead to increase susceptibility to infections. This is particularly true for approaches neutralizing the IFN-α protein itself. Similarly, blocking TLR7 and TLR9 by means of inhibitory ODN, could lead to inability to fight DNA and/or RNA viruses. However, recent data suggest a high level of redundancy between TLR and other nucleic acid specific cytosolic receptors in the immune response to viruses. Indeed, patients defective in key signalling molecules of the TLR pathways such as MyD88, IRAK-4 [23, 101, 102] or UNC93B molecule [38] showed a surprisingly mild phenotype. *In vitro* data showed that PBMC of these patients are completely unable to respond to synthetic TLR7 and TLR9 ligands but still produce normal IFN-α level in response to DNA and RNA viruses, probably using the alternative IFN-α/β-inducing pathways such RIG-1, MDA5 and DAI [102].

Conclusions

Our understanding of how human PDC respond to viruses and self ligands has greatly improved in recent years. Despite this, many questions remain to be solved; (i) what are the key molecules controlling the compartmentalization of IFN-α response in PDC; (ii) how do TLR7 and TLR9 sense their ligands and re-localize from the ER to the endosomes; (iii) are human PDC able to produce IFN-α via MYD88/TLR7/9 independent pathways?

It has become clear that IFN-α can have a key role in boosting the adaptive response whilst chronic over-production of IFN-α can be detrimental and leads to autoimmunity. This understanding has significantly broadened the list of diseases that would benefit from immunostimulatory drugs triggering TLR7 and TLR9 versus those that would benefit from drugs antagonizing these receptors, opening up an exciting future for therapeutic approaches targeting human PDC.

Conflict of interest statement

The authors are full time employees of Dynavax Technologies. The authors have no other conflicting financial interests.

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